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CAPTURE AND BOMA STRESS RESPONSES IN THE WHITE RHINOCEROS (*CERATOTHERIUM SIMUM*)

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A Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree Doctor of Philosophy.

Johannesburg 2017

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

_____ day of _____ 20_____ in _____

ABSTRACT

The translocation of rhinoceroses is extremely stressful to the animal and this may strongly affect the success of translocation. The objectives of this study in white rhinoceroses were to (i) validate a non-invasive assay using faecal metabolites to assess the stress response in the white rhinoceros, (ii) to assess the stress response in the white rhinoceros associated with capture, handling, transportation and confinement, to be able to predict at capture which animals will adapt to confinement, and (iii) to investigate the use of the acute phase proteins (APP), serum amyloid A (SAA) and haptoglobin (Hp) as indicators of stress associated with capture and confinement. To achieve these objectives blood and faecal samples were collected from each rhinoceros at capture, and whenever possible, during confinement. To assess the hypothalamic-pituitary-adrenocortical axis (HPA) response of white rhinoceroses an ACTH challenge was performed and plasma cortisol and faecal glucocorticoid metabolites (FGM) were measured with a commercially available ^{125}I RIA kit and a ^{125}I corticosterone RIA kit, respectively. Gastrointestinal transit time was estimated with the use of an inert marker. Results showed a 4-to 8-fold increase in plasma cortisol within 15 to 20 minutes and a 3-fold increase in FGM concentration 60 to 90 hours later. Although this FGM measurement is non-invasive, relevant and robust, the time required to complete the extraction and assay could take several days. This measurement can be advantageous to monitor the stress of animals in confinement but has no application where animals are captured and immediately translocated. Measurement of plasma cortisol and FGM was also used to assess stress in rhinoceroses following transportation and in confinement. The results show that rhinoceroses have variable individual responses to capture and confinement and although there was evidence of behavioural habituation, HPA activity showed that there was no physiological habituation. We could not establish any predictor of success of habituation in the boma environment. The APP results showed that Hp is more likely to be an indicator of metabolic stress; rather than physical and psychological stress; while SAA responds rapidly to physical and psychological stress in the rhinoceros. It was also found that plasma cortisol was positively

associated with SAA concentration when the animal is transferred to the boma, and that SAA may be a potential plasma biomarker to identify animals which could be at risk of failing to habituate to confinement.

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NOMENCLATURE

ACTH	Anterior pituitary adrenocorticotropic hormone
AUC	Area under curve
APP	Acute phase protein
APR	Acute phase response
CBG	Corticosteroid binding globulins
CRH	Corticotrophin-releasing hormone
CV%	Coefficient of variance
EIA or ELISA	Enzyme-linked immunosorbent assay
FGM	Faecal glucocorticoid metabolites
GC	Glucocorticoid
Hp	Haptoglobin
HPA axis	Hypothalamic-pituitary-adrenocortical axis
HPLC	High performance liquid chromatography
IM	Intramuscular
IV	Intravenous
KNP	Kruger National Park
RIA	Radioimmunoassay
SAA	Serum Amyloid A

LIST OF SYMBOLS

$IU.kg^{-1}$	International units per kilogram
g	Gram
$ng.g^{-1}$	Nanogram per gram
$ng.ml^{-1}$	Nanogram per millilitre
$nmol.ml^{-1}$	Nanomole per millilitre

CHAPTER 1

Introduction

Animal populations all over the world are declining as a result of human activity (Cooke et al., 2014) and human impacts or anthropogenic impacts. This includes impacts on the biophysical environment and biodiversity (Sahney et al., 2010; Hawksworth and Bull, 2007; Hutchins and Kreger, 2006). In this regard conservationists need to consider interdisciplinary approaches and find rationales to prevent loss in biodiversity in an attempt to facilitate recovery of endangered species. Two recent interfaces recognized in conservation are animal behaviour and physiology. Integrating these disciplines can generate meaningful data to support conservation management actions (Cooke et al., 2014). Management actions include population management through translocation and re-introduction (Armstrong and Seddon, 2008).

Translocation and re-introduction of animal species and more specifically of white rhinoceroses to other areas in Southern Africa is essential to establish multicentric populations in an attempt to save the species from being poached to extinction. In South Africa the poaching of mostly white rhinoceroses and some black rhinoceroses has increased from 0.03 rhinoceros per day in 2007 to 2.75 rhinoceros per day by the end of 2013 (Di Minin et al., 2015) and 2.95 rhinoceros per day by the end of 2016 (Savetherhino, 2017).

My particular interest was the white rhinoceros population in the Kruger National Park (KNP), which is considered one of the primary sources of healthy white rhinoceroses for translocation in Southern Africa. The KNP is situated in the north-eastern corner of South Africa, bordering Mozambique in the east and Zimbabwe in the north (22°19'and 25°32' latitude and 30°54' and 32°02' longitude) (Figure 1.1). The KNP covers an area of 1,948,528 hectares and was proclaimed as a National Park in 1926. In 1961, the first white rhinoceroses were re-introduced from the Umfolozi Game Reserve in Kwa-Zulu Natal to the KNP

after being almost extinct in the area. Over a twelve-year period a total of 345 white rhinoceroses were translocated to the KNP from Umfolozi and by 1993 the numbers had increased to 1875 (Pienaar, 1994). The numbers are currently estimated in the region of 8968; which is the single largest population



Figure 1.1 Location map of the Kruger National Park within Southern Africa.

of free-ranging southern white rhinoceroses *Ceratotherium simum* in South Africa (Ferreira et al., 2015). As Operations Coordinator for KNP Veterinary Wildlife Services, I was responsible for the planning and execution of rhinoceros captures and translocation operations and also for the supervision of the boma-training phase of the rhinoceroses. The large numbers of white rhinoceroses that are available for translocation all over Southern Africa from the KNP made the KNP the most suitable area to conduct a study of this nature.

The translocation of any animal species, but especially rhinoceroses, can be extremely stressful to the animal and this may strongly affect the success or outcome of the translocation. Stress should be considered as a predictable factor in translocation and should be integrated into planning these operations (Dickens et

al., 2010). The biggest factor for rhinoceroses when translocated is the enormous change from its natural environment to the destination environment (Turner et al., 2002). Six confinement-specific stressors are considered to be important in the case of the rhinoceros; restricted movement, absence of retreat space, forced proximity to humans, routine husbandry, restricted feeding and foraging opportunities, and abnormal social groups. These potential stressors all have one common denominator; the fact that the captive animal does not have the ability to control any of them (Morgan and Tromborg, 2007). The confinement phase of translocation however forms a very important part of the adaptation period (boma-training) for the rhinoceros in which these animals are introduced to cultivated fodder such as lucerne *Medicago sativa* and *Eragrostis spp*. grass. The rhinoceroses seem to habituate to confinement, both behaviourally and hormonally, as the intensity of the response to a repeated set of stimuli due to confinement gradually declines, as also noted in other species (Cyr and Romero, 2009). Boma-training is also essential when white rhinoceroses are translocated to areas where vegetation is scarce during certain periods of the year, as they do not readily take to cultivated fodder if they were not gradually introduced to it.

Rhinoceroses, like other animals, differ in their ability to cope with the changes associated with translocation. Certain individuals do not adapt to captive conditions, presumably because they experience the consequences of being chronically stressed. This situation can be caused by a number of acute stressors and may necessitate the rhinoceroses being released back into their natural habitat, before serious health-related problems occur. It is therefore important to be able to evaluate the levels of stress (over time) in the animals during the adaptation/habituation period or even before they go into captivity.

Evaluation of the stress response to the initial capture phase can be made from blood samples obtained from the immobilized animals. However, once the rhinoceroses are released into the boma there is no longer any opportunity to obtain blood samples, thus the application of a non-invasive assessment of the stress response was essential to this study. Being able to predict early-on in the

period of boma-confinement which animals were likely to adapt and which were not, based on some measured physiological variable, was also a primary objective of the study.

1.1 Objectives

The objectives of this study can be summarized as follows:

i) The first major objective was to validate, for white rhinoceroses, a technique to measure the adrenocortical activity using a faecal glucocorticoid metabolite (FGM) assay that has previously been used in several other species. Included in this objective was the determination of gut transit time, through the use of a digestive marker, since an elevation of the plasma glucocorticoid hormone (cortisol) may precede the appearance of the excreted metabolites by a significant time. Further objectives were to quantify the effects of delayed sample processing and storage times on the results.

ii) The second major objective was to use blood samples obtained from white rhinoceroses at capture and again at subsequent release into the boma to assess the stress of capture, handling, confinement and transport experienced by white rhinoceroses.

iii) The third major objective was to determine, from either the FGM or the blood concentration of stress hormones obtained at capture, whether they can be used to predict which animals will adapt to confinement and begin to feed and those that will not.

iv) The fourth objective developed during the course of the study was to make use of blood samples obtained from boma-habituated white rhinoceroses to investigate whether serum amyloid A and haptoglobin could be indicators of the stress of capture, handling and transportation in white rhinoceroses.

The methods, results and conclusions to these studies appear in the following chapters:

Chapter 2 describes the animals used in the study and the procedures they underwent regarding their capture and confinement in the bomas. This chapter also describes the use of four boma-adapted animals for specialist investigations.
Chapter 3 describes the use of an ACTH challenge to validate faecal sample assessment in white rhinoceroses.
Chapter 4 describes the Hypothalamic-Pituitary Axis responses to capture and boma confinement in white rhinoceroses.
Chapter 5 describes the investigation of Serum Amyloid A and Haptoglobin as indicators of the stress of capture, handling and transportation in white rhinoceroses.
Chapter 6 concludes by summarising the results obtained in this study and recommendations are made regarding future research on the evaluation of stress in the white rhinoceros.

1.2 Literature review

1.2.1 Stress

In recent times, much research has focused on ways to identify chronically stressed wild animals in the light of all the anthropogenic disturbances. The most common way to do this is to measure the glucocorticoid levels (Munck et al., 1984; Sapolsky et al., 2000; Holst, 1998; Romero, 2004; Korte et al., 2005; Wingfield and Sapolsky, 2003; Wingfield et al., 1992; Romero, 2002; Mostl and Palme, 2002; Touma and Palme, 2005) with the assumption that levels are increased in chronically stressed animals. Literature, however, provides very little guidance on how wild animals will hormonally respond to stressors that cause chronic stress. Most of the available literature lacks empirical data and has to resort to theoretical models (Dickens and Romero, 2013).

There are three major stressors to all vertebrates in nature namely, starvation or malnutrition, adverse environmental conditions and predation attempts (Romero et al., 2009). The term “stress” was introduced in 1935 by Hans Selye, who defined

the term or phenomenon as “nonspecific bodily changes that occur in response to harmful stimuli or stressors”. Stress is also defined as the sum of biological reactions to intrinsic and extrinsic stimuli that result in a perturbation from homeostasis (Chrousos and Gold, 1992). Levine (2005) emphasized that stress should be considered as a process that includes the stimulus, the perceptual processing of the input and the behavioural and physiological output. Cockrem (2013) defined stress as “the state when the hypothalamo-pituitary-adrenal (HPA) axis is activated with increased secretion of glucocorticoids in response to a stressor”.

The process involved to help the body maintain homeostasis which is controlled by the emotional brain is termed allostasis, which means “stability through change” (Sterling, 2004; McEwan and Stellar, 1993). Allostasis involves invoking mechanisms which change the controlled physiological variable; through mediators of allostasis; by predicting what level of these mediators will be needed for the anticipated demand. The mediators of allostasis include adrenal hormones, neurotransmitters and cytokines (Korte et al., 2007). Romero et al. (2009) propose that allostasis is the process of maintaining homeostasis through changes in physiological mechanisms in response to changes in energetic demands and reactive scope. Koolhaas et al. (2011) defines a stressor as a stimulus from which the “response demands” exceed the adaptive capacity of an organism.

Response to stress works through receptors in various organs and tissues to produce changes that are adaptive to metabolism, the immune system and the cardiovascular system (Korte et al., 2007). Adaptation to physical and psychological stress usually involves the release of hypothalamic corticotrophin-releasing hormone (CRH), followed by anterior pituitary adrenocorticotropic hormone (ACTH) and the adrenal glucocorticoids (GCs), namely cortisol and/or corticosterone by the HPA axis into systemic circulation (Dallman et al., 1994; Haussmann et al., 2007; Romero, 2004) (Figure 1.2). Circulating steroid hormones are then metabolized by the liver and excreted via the kidneys into the urine or via the bile into the gut. The excretion via the bile route creates a lag time

when comparing plasma and faecal cortisol, is species-specific and when taken into consideration a similar cortisol pattern is found in the faeces and in the plasma. Faecal cortisol is, however, less affected by episodic fluctuations or the pulsatility of hormone secretion because it is integrated over a certain time period (Touma and Palme, 2005).

Stressors such as anaesthesia, handling, confinement, novel environments, and pain lead to elevated blood concentration of GCs in animals. This level of GC however typically decreases back to normal levels in time, as a consequence of habituation (Turner et al., 2002; Franceschini et al., 2008; de Kloet et al., 2005; Hill and Broom, 2009; Romero, 2004). The adaptation occurs through negative feedback to the brain by the GCs themselves to turn off the initial steps of the HPA. If there is a deficit in the efficiency of the negative feedback, elevated GC levels occur for a longer period, resulting in negative effects on the animal, such as lack of adaptation and failure to eat (Romero, 2004) (Figure. 1.2). It has been proposed that chronic stress and/or lack of adaptation has widespread negative effects on numerous physiological systems, including reproduction, growth, metabolism, immune function and behaviour, by stimulating endocrine responses (Powell et al., 1967a; Breazile, 1987; Morley et al., 1991; Harbuz and Lightman, 1992; Stratakis and Chrousos, 1995; Wingfield and Sapolsky, 2003; Berga, 2008; de Kloet, 2003; Wikgren et al., 2012). Human and laboratory animal studies have indicated that chronically elevated levels of the stress hormone cortisol are associated with anorexia and depression (Sapolsky et al., 2000; Brambilla, 2001).

During stressful periods the release of GCs can meet the increased metabolic demands of the body. The availability of GCs are however regulated, in part, by corticosteroid-binding globulins; where any free or unbound GCs can enter cells and allow energy mobilization to fuel fight-or-flight responses (Mendel, 1989). Stress is therefore not always negative but can have a positive effect by making energy available to an animal at critical moments (Goymann et al., 1999; Jacobson, 2005). In situations of mild stress or stress of a short duration the animal makes use of reserve biological resources which can be replenished within

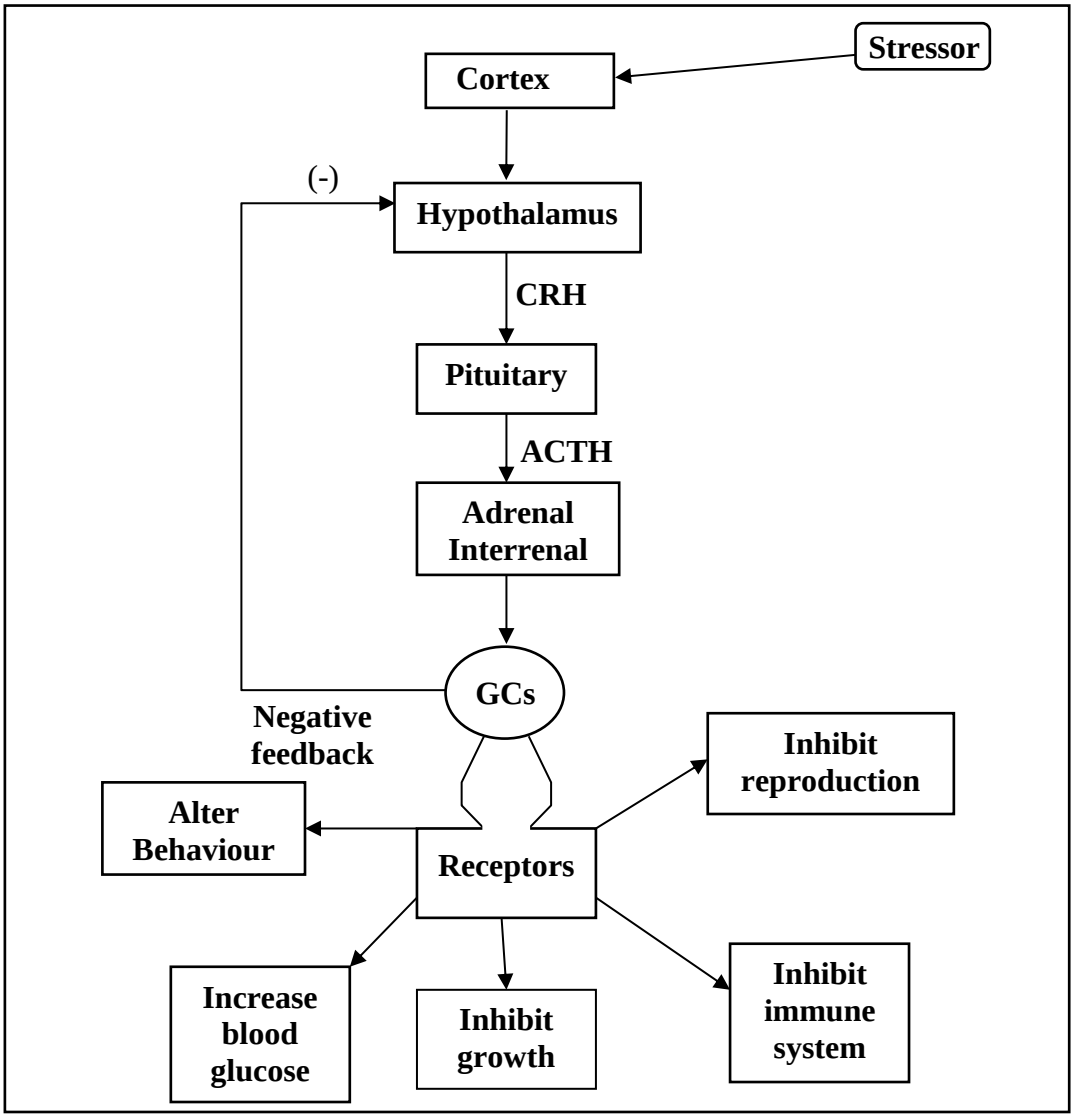


Figure 1.2 The hypothalamic-pituitary-adrenal (HPA) axis. An acute stressor is detected by the cortex of the brain, which sends a neuronal signal to the hypothalamus. The hypothalamus then sends a hormonal signal (CRH) to the pituitary, resulting in the pituitary sending a hormonal signal (Adrenocorticotropin, or ACTH) to the adrenal or interrenal gland (depending on the species) to release glucocorticoids (GCs). GCs have multiple effects that are mediated by blood-borne carrying proteins (Corticosteroid Binding Globulins, CBG) and two different receptors (Type I and II). A negative feedback loop shuts off the HPA pathway leading to GC release. If the stressor persists and the GCs remain elevated, negative feedback ceases to function and the deleterious chronic effects of GCs begin (adapted from Romero, 2004).

a short recovery period when homeostasis is re-established (Moberg, 2000). On the other hand, high stress levels (chronically elevated plasma GCs) for a prolonged period can have detrimental effects on an animal's wellbeing. This includes effects on the animal's immune function, which may then lead to immuno-suppression (Mostl and Palme, 2002; Peristein et al., 1993; Sapolsky et al., 2000). Therefore, the longer the animal secretes glucocorticoids, the greater the susceptibility to infections (Romero et al., 2009). GCs have primarily permissive and stimulatory roles in metabolism and behaviour, and primarily suppressive roles in the immune system (Sapolsky et al., 2000; Pruett, 2003).

Animals differ in their ability to cope with stress and the term used to describe this difference in ability is "coping style". Animals cope with changing environments by using both behavioural and physiological stress responses (Budzyńska, 2014). An animal's coping style is shaped evolutionarily as adaptations to aversive situations; it is also a behavioural reaction and has a reducing effect on stress. If aversive conditions which an animal is confronted with are entirely different to those found in its natural environment it may not have the adaptive behavioural response to cope with the situation, this may then lead to negative outcomes (Wechsler, 1995).

The physiological response to a stressor may be identified or characterized by monitoring the appearance of the hormonal mediators, GCs and catecholamines in the blood. The increased GCs appear within several minutes with the duration of elevated GCs depending on the nature and duration of the stressor (Armario, 2006; Cockrem, 2013). Research on lab rats showed that a single exposure to stress caused an increase in the plasma levels of HPA hormones and caused long-term desensitization of the response of the HPA axis (Belda et al., 2004; Belda et al., 2008). Further research showed that when the same stressor is applied to animals for a prolonged period of time the stress response slowly diminishes leading to habituation (Dhabhar and McEwen, 1997). A bidirectional interaction furthermore exists between hormones and behaviour, where hormones can affect

behavioural response and behaviour can influence hormone levels (Budzyńska, 2014).

Under conditions of confinement, non-invasive assessment of the stress hormone levels through the faeces is a viable alternative to determining the stress hormone levels through blood (Bayazit, 2009). This alternative is considered appropriate since repeated capture, restraint and blood sampling would be impractical as these interventions themselves elicit a stress response and increase the risk of mortality (Romero, 2004; Sapolsky, 1982; Touma and Palme, 2005; Wingfield et al., 1992). Circulating GCs are metabolized in the liver and excreted in the urine (via the kidneys) and faeces (via the bile) into the small intestine. Significant correlations have in the past been demonstrated between serum glucocorticoids levels and faecal glucocorticoid levels (Palme and Möstl, 1997; Touma and Palme, 2005; Wasser et al., 1997; Whitten, 1997).

Measurement of FGM is a technique already widely used to assess stress in wildlife (Keay et al., 2006; Millspaugh and Washburn, 2004; Schwarzenberger, 2007; Touma and Palme, 2005; Von der Ohe and Servheen, 2002). Previous species-specific studies include work on roe deer (Dehnhard et al., 2001), spotted hyena (Goymann et al., 1999), red deer (Huber et al., 2003), Steller sea lion (Hunt et al., 2004), grizzly bear and African elephant (Hunt and Wasser, 2003), felid species (Graham and Brown, 1997), northern spotted owl (Wasser et al., 1997), African elephant (Foley et al., 2001; Millspaugh et al., 2007; Stead et al., 2000; Viljoen et al., 2008), wolves and elk (Creel et al., 2002), tufted capuchin monkeys (Lynch et al., 2002), tammar wallaby (McKenzie and Deane, 2005), big horn sheep (Miller et al., 1991), Grevy's zebra (Franceschini et al., 2008), white tailed deer (Millspaugh et al., 2002; Washburn and Millspaugh, 2002), wild dog (Monfort et al., 1998), Chacma baboons (Weingrill et al., 2004) and other carnivores (Young et al., 2004).

Research on measurement of glucocorticoid levels has also been conducted on several rhinoceros species and started as early as 1990 (Kock et al., 1990). One of

the first ground-breaking studies on black and white rhinoceroses was done by Brown et al. (2001) followed by Turner et al. (2002) and Carlstead and Brown (2005) also on black and white rhinoceroses. More recent research was done by Linklater et al. (2010) on black and white rhinoceroses, Metrione and Harder (2011) on white rhinoceroses, Santymire et al. (2012) on black rhinoceroses and Capiro et al. (2014) on Indian rhinoceroses.

Kock et al. (1990) measured some biological parameters including cortisol concentrations when they translocated black rhinoceroses. Their results showed a significant difference in cortisol concentrations in three blood samples collected respectively at capture, transportation to a holding facility and after a period of confinement, where the second sample showed the highest concentration depicting the stress associated with the capture.

Brown et al. (2001), in their study on zoo animals, showed that there was no difference in faecal cortisol concentrations between male and female rhinoceroses and that the means were significantly higher ($P < 0.05$) in black than in white rhinoceroses; $41.8 \pm 3.1 ng.g^{-1}$ and $31.2 \pm 1.7 ng.g^{-1}$ respectively.

Research done by Turner et al. (2002) showed that in captivity-adapted white rhinoceroses faecal cortisol concentrations ranged from 2.0 to $7.3 ng.g^{-1}$ dry faeces and corticosterone from 4.0 to 10.8 $ng.g^{-1}$ dry faeces. The research also showed higher levels of faecal cortisol and corticosterone associated with the combined effects of restraint and translocation; 6.9-to 10-fold and 3.2-to 4.5-fold respectively in both black and white rhinoceroses. Furthermore, their research showed that there is a significant decrease in both faecal cortisol and corticosterone after release following translocation in black rhinoceroses. The FGM concentrations in this study were lower than that of the study by Brown et al. (2002). Although zoo animals were used in both studies, different methods and assays used account for the different results in the studies.

Carlstead and Brown (2005) found that corticoid variability appears to be an indicator of chronic stress caused by social stressors in captive black and white rhinoceroses. Linklater et al. (2010) also showed elevated corticoid levels of two to five fold after 17 days in confinement after capture, with a decline with longer time in captivity in both black and white rhinoceroses. Metrione and Harder (2011) measured concentrations of metabolized corticosterone in female white rhinoceroses and reported that there was no difference in concentrations between dominant and subordinate sub-adult females. The way females were kept in captivity however showed differences in GC levels in different age groups of females whether they were housed individually or with an unknown companion or with a known companion; the latter tended to be associated with a lower mean corticosterone concentration. Furthermore, wild-captured females had a higher average corticosterone concentration than captive-born females. Santymire et al. (2012) used ACTH challenges to validate a corticosterone enzyme immunoassay (EIA) to assess adrenocortical activity in African wildlife species including black rhinoceroses. Their work showed there is a surge in FGM 75 hours after the ACTH injection.

Capiro et al. (2014) performed an ACTH challenge on an adult male Indian rhinoceros which resulted in a 38-fold and 3.5-fold increase in urinary glucocorticoid metabolites and FGM respectively. Their work on the same species also showed that the mean and peak FGM concentrations differed among individual females, but that all had elevated concentrations after translocation that lasted up to nine weeks.

These non-invasive studies not only concluded that FGM can be used successfully to determine adrenal activity in wildlife, more specifically rhinoceroses, but also, refined sample preservation methods, extractions, assays and validation procedures. The faecal levels of GC metabolites also indicate a cumulative response over time, rather than the instantaneous response indicated by the blood hormone concentration. The use of FGM to detect stress in animals is, however,

time consuming and results are only obtained days after sampling but it is definitely of value when monitoring animals in confinement.

1.2.2 Acute Phase Response

Acute phase response (APR) proteins were also investigated as an alternative to FGM to quantify the stress of capture and handling in white rhinoceroses. Studies on humans, rodents and domestic animals have highlighted the role of pro-inflammatory cytokines in response to psychological stress (Hale et al., 2003; Kang and Fox, 2001; Nukina et al., 2001). These cytokines are secreted by activated macrophages and modulate protein synthesis by the hepatocytes. These proteins are acute phase proteins (APP) and are secreted in response to a stressor in the so-called APR to re-establish homeostasis and promote healing as part of the innate immune response (Carroll and Forsberg, 2007). APP can be grouped in positive APP, which increase in response to the APR, and negative APP, which decrease in response to the APR. Positive APP can further be classified as major, moderate and minor APP according to the magnitude of their increase in the APR (Cray, 2012). Positive APP have multiple functions which include modulation of the immune system, protein transport and tissue protection from damage by the inflammatory process (Cray et al., 2009). Serum concentrations of APP return to basal when the triggering factor is removed (Petersen et al., 2004). Currently APP concentrations are used in veterinary medicine to assist in prognosis of diseases in companion and domesticated animals (Cray et al., 2009), furthermore they are also used as biomarkers of stress and indicators of stress (Cray, 2012).

Stress is known to raise plasma cortisol concentrations in several species (Nagata et al., 1999; Saeb et al., 2010; Schmidt et al., 2010), resulting in stimulation of the APR which cause an increase in tumour necrosis factor (TNF)-α and Hp in cattle (Cooke et al., 2012) and Hp and SAA in horses (Casella et al., 2012). Measurement of APP is considered to be a rapid diagnosis to evaluate an animal's welfare under stressful conditions (Gómez-Laguna et al., 2011). In this study, we

investigated the effects of stress associated with capture and confinement on the plasma concentrations of SAA and Hp in white rhinoceroses.

CHAPTER 2

Methods

At the core of this study are (i) experiments conducted on white rhinoceroses that had been confined to boma conditions for up to 120 days, and (ii) the collection of blood and faecal samples from white rhinoceroses that were captured in the wild and then confined to the bomas for an adaptation period. This chapter consolidates the procedures applied to each group of rhinoceroses to avoid unnecessary repetition of these details in the following chapters.

2.1 Boma-adapted animals

Four white rhinoceroses previously captured in the wild (Table 2.1) were used in a cross-over protocol to investigate blood and FGM concentrations in response to an ACTH challenge. The animals had been held separately in individual bomas in Skukuza for at least four months prior to the trial, were eating and defecating normally and were considered to be healthy based on behaviour, body condition, clinical examination and normal hematology.

Table 2.1 Information of white rhinoceroses used in ACTH challenge.

Animal	Sex	Age (year)	Weight (kg)
A	Male	20	2060
B	Male	8	1800
C	Female	6	1350
D	Female	6	1300

To conduct the ACTH challenge trial, the white rhinoceroses were immobilized between 7:00 and 10:00 in the morning with a drug combination consisting of etorphine hydrochloride (3-4 mg) (M99®, Novartis, P.O. box 92, Isando, Johannesburg, 1600, South Africa) and azaperone (40 mg) (Stresnil, Janssen

Pharmaceuticals Ltd., Halfway House, 1685, South Africa). This dose was delivered remotely into the nuchal hump (musculus rhomboideus cervices) using a 3.0 ml plastic dart with a 60 mm un-collared needle propelled by a compressed air rifle (DAN-INJECT International S.A., Private Bag X402, Skukuza, 1350, South Africa).

Once the rhinoceros was recumbent, an intravenous catheter was inserted into an auricular vein to allow for blood sample collection in evacuated tubes. Butorphanol (Kyron Laboratories, P.O. Box 27329, Benrose, 2011, South Africa) at a dose of 30-40 mg was administered intravenously (IV) immediately after the animal became recumbent, to improve cardiovascular and respiratory function under immobilization. Once the animal was stabilized after darting the first blood sample was collected and was designated as time zero (T0). Further samples were collected at five minute intervals up to 100 minutes. An ACTH analogue (Synacthen®, Novartis Pharma AG, CH-4002, Basel, Switzerland) or an equivalent volume of saline was administered to each immobilized rhinoceros ten minutes after collection of the baseline blood sample. The ACTH was administered intra-muscularly (IM) at the recommended dose of 1.25-1.5 IU/kg (Wasser et al., 2000). Blood samples were kept in ice water until sampling was completed. Serum and plasma were harvested by centrifugation and aliquots were kept at -80°C until the assays were performed. Fourteen days later the procedure was repeated, so that each animal had received either the Synacthen® or saline on each occasion.

After completion of all the procedures, the immobilization was reversed with 80-100 mg naltrexone (Naltrexone, Kyron Laboratories, P.O. Box 27329, Benrose, 2011, South Africa) (Miller et al., 2013).

2.2 Capture, transport and confinement trial

This study was conducted in the Kruger National Park (KNP), South Africa, on a wild population of white rhinoceroses which was authorized for translocation

within South Africa during 2008 to 2012. For this study 124 white rhinoceroses were immobilized from a helicopter using a drug combination consisting of 2-4 mg etorphine hydrochloride (M99®, Novartis, Kempton Park, South Africa) with 20-40 mg azaperone (Stresnil®, Jansen Pharmaceuticals Ltd, Halfway House, South Africa) and 5,000 IU hyaluronidase (Hyalase, Kyron Laboratories, Benrose, South Africa); this dose was also dependent on the size of the rhinoceros. The dose was delivered into the gluteal muscle using a 3.0 ml plastic DAN-INJECT dart with a 60 mm un-collared needle propelled by a compressed air rifle (DAN-INJECT, International S.A., Skukuza, South Africa).

Butorphanol (Kyron Laboratories, Benrose, South Africa) was administered intravenously immediately after the rhinoceroses became recumbent at a dose of 40-80 mg, depending on the amount of etorphine used, to improve respiratory and cardiovascular variables (Miller et al., 2013). Blood samples were then collected from the auricular vein. Once all further data were collected an electric cattle prodder was used to stimulate the blindfolded rhinoceros to stand up and be guided into a transport crate. In the crate the rhinoceros received a partial antidote of diprenorphine (M5050®, Novartis, Kempton Park, South Africa) intravenously at three times the amount of etorphine used in the dart as well as 50-100 mg zuclopenthixol acetate (Acuphase®, H. Lundbeck A/S, Ottiliavej 9, DK – 2500, Valby, Denmark) for extended tranquilization. The crate was then loaded onto a vehicle and transported to the boma complex in Skukuza (transport time approximately 60 minutes). A further blood sample was then obtained before the rhinoceros received 80-100 mg naltrexone (Naltrexone, Kyron Laboratories, P.O. Box 27329, Benrose, 2011, South Africa) to reverse the effects of the etorphine. It also received an additional 40-80 mg azaperone for tranquilization. The rhinoceros was then released into the introduction boma (see Figure 2.1). A further blood sample was collected from each animal before it was loaded for either release due to failure to adapt (10-12 days later) or translocation following successful adaptation (120-180 days later). Failure of animals to adapt was subjectively determined when the animals did not eat or defecate within 10 to 12 days after capture, as these animals may have become physiologically

compromised. Blood samples were kept in ice water and transported to the laboratory for processing. Serum and plasma were harvested by centrifugation and aliquots were kept at -80°C until the assays were performed.

All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC no. 2008/22/05) and the Animal Use Committee of the South African National Parks.

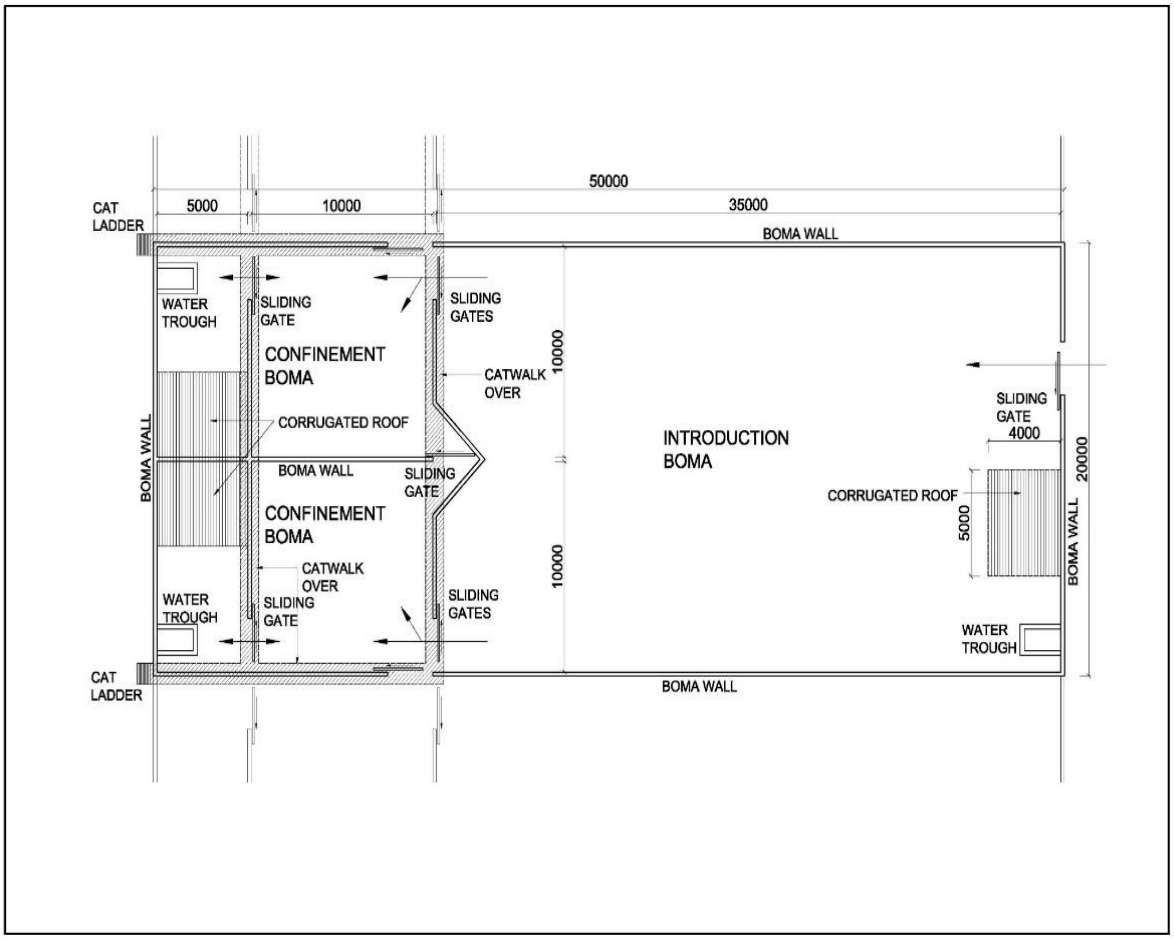


Figure 2.1 Boma layout on a scale of 1:200. Arrows indicate direction of movement of rhinoceroses during period of confinement.

CHAPTER 3

The adrenocortical response of wild white rhinoceroses to capture and confinement; use of an ACTH challenge to validate faecal sample assessment

To evaluate the stress associated with the capture and boma-habituation of free-ranging white rhinoceroses *Ceratotherium simum* from the Kruger National Park (KNP) (South Africa), we determined whether non-invasive assessment of faecal stress hormone metabolites (Wasser et al., 2000) is a reliable tool.

Non-invasive measures such as FGM measurements are a useful alternative for monitoring stress levels as the animals do not have to be captured for sampling (Romano et al., 2010). FGM concentrations also fluctuate less over time in response to circadian rhythms and pulsatile secretions of cortisol (Keay et al., 2006). In evaluating FGM concentrations it is important to account for the lag time between the secretion of cortisol due to a stressful event and the appearance of FGM (Palme, 2005).

The aim of the study was to stimulate adrenocortical activity, to raise the concentration of circulating cortisol and then measure the resulting concentration of FGM. In addition, the reliability of this method was established by evaluating faecal samples for the effects of environmental decay, extraction efficiency and intra-sample variation.

The capture, boma-habituation and translocation of free-ranging white rhinoceroses from the KNP has become an essential tool to establish new breeding groups and to reduce the impact of poaching (Ferreira et al., 2012). Translocation will also ensure that multicentric populations are established for the long-term survival of the species (Dickens et al., 2010). The capture of rhinoceroses in the field presents an initial acute stress, which may be followed by chronic stress after being placed into holding facilities (bomas) (Dickens et al., 2010; Cyr and Romero, 2009). The housing of animals in bomas for weeks to months at a time is

an essential part of the acclimatization and habituation process that is necessary before translocation.

During this period of boma-confinement the rhinoceroses are introduced to alternative food sources and presumably become desensitized to the stressors associated with captivity. Rhinoceroses differ from each other individually in their ability to cope with the stressors, both acute and chronic, which may result in lack of adaptation to captive conditions (personal observation). Individual animals may become chronically stressed as a result of numerous acute stressors, such as nutritional stress, physiological stress and psychological stress (Dickens et al., 2010). Chronically stressed rhinoceroses may become physiologically compromised, at which time they may need to be released back into their natural habitat before serious health-related problems occur. Examples of possible health concerns may include immunosuppression and increased susceptibility to secondary infections (Carlstead and Brown, 2005; Kock et al., 1999; Moberg, 2000; Mostl and Palme, 2002; Pride, 2005; Roth and Vance, 2007; Vance et al., 2004). It is therefore important to be able to evaluate the levels of stress over time in the animals during the adaptation period.

A change in plasma glucocorticoid concentration (GC) is reported to be an effective index of a stress response to adverse stimuli. In stressed animals the hypothalamic-pituitary-adrenocortical axis (HPA axis) is activated resulting in the release of cortisol mediated by adrenocorticotropic hormone (ACTH) (Goymann et al., 1999; Touma and Palme, 2005). Studies in the laboratory rat have shown that adaptation to stress is associated with a return of plasma GC to basal concentrations because of negative feedback (Windle et al., 1998). In chronically stressed animals the negative feedback is however disrupted and these animals experience prolonged elevated GC concentrations (Dallman et al., 1992). A study on both black and white rhinoceroses showed that the capture and translocation processes caused elevated levels of cortisol in serum, urine, and faeces and that these levels are several-fold higher than those in animals that have adapted to a new environment six weeks after translocation (Turner et al., 2002).

The immobilization of animals for blood collection can result in a stress response with increases in plasma cortisol concentrations (Romero, 2004; Sapolsky, 1982; Touma and Palme, 2005; Wingfield et al., 1992). Because immobilization for blood sampling may elevate plasma cortisol concentrations, non-invasive measures such as FGM measurements offer a useful alternative for determining stress levels as the animals do not have to be captured for sampling (Romano et al., 2010). Also, FGM concentrations fluctuate less over time in response to circadian rhythms and pulsatile secretions of cortisol (Keay et al., 2006). In evaluating FGM concentrations it is important to account for the delay time between the secretion of cortisol due to a stressful event and the appearance of FGM (Palme, 2005). The gastrointestinal transit times in different species and their unique physiology lead to variations in this interval (Keay et al., 2006). Once the delay in timing is understood, the relationship of FGM and plasma cortisol can be elucidated (Touma and Palme, 2005).

3.1 Materials and methods

3.1.1 Animal subjects

Four white rhinoceroses captured in the wild (see Chapter 2) were used in a cross-over protocol to investigate blood cortisol and FGM concentrations in response to an ACTH challenge.

3.1.2 Sample collection and ACTH challenge

Once the rhinoceros was recumbent after immobilization, an intravenous catheter was inserted into an auricular vein to allow for blood sample collection in evacuated tubes. Once the animals were stabilized the first blood sample was collected and was designated as time zero (T0). Further samples were collected at five-minute intervals up to 100 minutes. ACTH or saline control was administered to each immobilized rhinoceros ten minutes after collection of the baseline blood sample (T0). The saline or the ACTH analogue (Synacthen®, Novartis Pharma

AG, CH-4002, Basel, Switzerland) was administered intra-muscularly at the recommended dose of 1.25 - 1.5IU.kg^{-1} (Wasser et al., 2000). Administration of the saline control and the Synacthen® was randomized in a cross-over protocol with two weeks between each trial.

To confirm that an increase in the plasma cortisol concentration produced a detectable change in the levels of FGM, faecal samples were collected daily for analysis. A fresh faecal sample was collected in the morning from the enclosure of each animal for seven consecutive days prior to each trial. An additional fresh faecal sample was also collected from the rectum of each animal at the time of immobilization (baseline). Following each trial, a faecal sample was then collected immediately from each episode of defecation, both day and night, for a further seven days. All faecal samples were mixed well and approximately 100g of each was frozen at -20°C until the FGM extractions were performed.

To establish at what time an induced increase in plasma cortisol concentration is reflected in the FGM levels in the faeces, the gastrointestinal transit time was determined (Palme, 2005). This transit time was estimated with the use of an inert marker introduced into the stomach of each rhinoceros at each trial. The inert marker was 20ml non-toxic glitter that was suspended in 40ml of corn syrup (Brown et al., 2001; Kruger et al., 2011). The marker was administered using a gastric tube, just prior to reversing the immobilization with 80-100mg naltrexone (Naltrexone, Kyron Laboratories, P.O. box 27329, Benrose, 2011, South Africa). An aliquot of each faecal sample that was collected for the following seven days was thoroughly inspected for the presence of glitter. The sample was examined for the presence of glitter under a dissecting microscope. Gastrointestinal transit time was confirmed from the first sample in which the highest number of glitter particles was detected (Steuer et al., 2010). The remaining portion was stored for the FGM analyses.

To confirm that a detectable increase in FGM could be detected in wild-captured animals fresh faecal samples were collected from the rectum of five rhinoceroses

immobilized in the wild (see Chapter 2 for details). The FGM levels of these samples were then compared to a fresh sample obtained from the enclosure of each animal after 5-6 days of subsequent boma-confinement.

3.1.3 Plasma cortisol

Plasma cortisol was measured using a commercially available ^{125}I RIA kit (Coat-a-Count®, Siemens Healthcare Diagnostics, Los Angeles, Santa Ana, CA 90045, USA).

3.1.4 FGM estimation and assay validation

Extraction of FGM from faecal samples: In preparation for the FGM extraction, each faecal sample was thawed at room temperature and mixed thoroughly. A sample of 2.5g (± 0.001g) was taken from the mixture. This sample was placed in a 50ml conical tube with 10ml 80% methanol (Palme et al., 2013), rotated at room temperature on a vertical plane for 16 hours and then centrifuged at 1,000 x g for 10 minutes. The supernatant was then withdrawn and stored at -70°C. The extraction technique was modified from previous reports (Brown et al., 2001; Touma and Palme, 2005; Wasser et al., 2000) as rhinoceros faeces have very coarse material with large stick-like particles. Instead of trying to remove this coarse particulate matter and then pulverizing the sample, a larger faecal sample was taken and simply mixed with methanol for 16 hours. Our preliminary research demonstrated that the 2.5g of faeces, instead of 0.5-1.0g of faeces, improved the coefficient of variation (CV%) of repeated extractions from 12% to 7%. To establish the dry weight of each faecal sample, an identical 2.5g of the sample was placed in a foil cup and dried in an oven at 60˚C for 48 hours. The dry matter weight of the faeces was then recorded.

Assay of FGM in faecal extracts: The measurement of FGM was performed with a ^{125}I Corticosterone RIA kit (MP Biomedicals LLC, Santa Ana, CA 92707, USA) that has been validated for several species including South African herbivores

(Brown et al., 2001; Carlstead and Brown, 2005; Chinnadurai et al., 2009; Wasser et al., 2000). The methanol extracts were diluted 2:1 with the manufacturer's steroid diluent (Cat. No. 07-166196) and assayed according to instructions. Except for one important consideration, which was a critical detail noted by my supervisor Dr. Pitts. The six corticosterone calibrators in the kit (0-1000ng.ml^{-1}) are pre-diluted to account for the 1:200 dilution prescribed for rat plasma corticosterone measurement. This means that the actual concentration of each calibrator is 200 times lower than stated, so our calibration range extended from 0 to 5ng.ml^{-1}. The FGM concentrations determined were then corrected for dilution ratios and water content of the faeces and the results expressed as ng.g^{-1} dry faeces. Serial dilutions of selected samples gave slopes of $r^2 > 0.98$ which remained parallel to the standard curve.

Environmental exposure: To investigate the effect of environmental exposure on the FGM content of the sample, fresh rectal faecal samples were obtained using pooled collected faeces from five different immobilized rhinoceroses of different ages and sexes. The samples were mixed well together and one aliquot was immediately frozen at -20°C until further analysis. The remainder of the sample was left on the ground and exposed to direct sunlight for a further four hours. Representative samples were then obtained from the center of the bolus at times 60, 120, 180 and 240 minutes of environmental exposure. These samples were then also frozen at -20°C until further analysis.

Sample storage: To investigate the effect of duration of freezer storage on the FGM levels, fresh faecal samples were obtained from five rhinoceroses immobilized in the boma. Each sample was mixed well and one aliquot of each sample was immediately processed for extraction of the FGM. A further aliquot was kept in a refrigerator at 4°C for 24 hours before being extracted. The remainder of the sample was divided into aliquots and immediately frozen at -20°C until further analysis. These aliquots were then extracted after one day, three days, one week, one month and nine months of storage.

Faecal cortisol: The presence of any cortisol immune-reactivity in the faecal extracts was also assessed using a ^{125}I RIA cortisol kit (Coat-a-Count®, Siemens Healthcare Diagnostics, Los Angeles, CA 90045, USA) (Brown et al., 2001).

Accuracy of the FGM measurement: Possible sources of variation in this methodology include the within and between assay variation in the FGM concentration (ng.ml^{-1}) measured in the supernatants, and the efficiency and repeatability of the extraction of the FGM from the faecal samples (ng.g^{-1} dry faeces). To investigate the within assay variation, the FGM concentration in a methanolic extract from a single faecal sample was measured seven times in the same assay. Also, to investigate the between assay variation, the FGM concentration (ng.ml^{-1}) in a methanolic extract from a single faecal sample was measured in duplicate in five different assays. To investigate the repeatability of the extraction method, the FGM concentration (ng.g^{-1} dry faeces) was determined from six aliquots of the same faecal sample in the same assay. To investigate the efficiency of the extraction method aliquots of the same faecal sample were spiked with a known quantity of corticosterone (Sigma cat. C2505; USA) before the addition of the methanol. Percentage recovery was calculated from the observed and expected FGM concentrations.

3.1.5 Data analysis

All data are represented as mean ± s.d. where applicable. Data were statistically analyzed using the GraphPad Prizm 5.03 software program (www.graphpad.com). Responses over time were subjected to repeated measures ANOVA at $p < 0.05$ with Bonferroni's correction. To evaluate the plasma cortisol response to Synacthen®, the area under the curve (AUC) was calculated using the GraphPad software. A paired t-test at $p < 0.05$ was used to compare (i) gastrointestinal transit time in the saline and ACTH administrations, and (ii) FGM levels at capture and after boma confinement.

3.2 Results

3.2.1 Plasma cortisol

Figure 3.1 (left panel) shows plasma cortisol concentration over time in the four boma-habituated rhinoceroses (A, B, C and D) in response to administration of Synacthen® or saline. Administration of Synacthen® induced a significant increase (4- to 8-fold) of the plasma cortisol with a statistically significant increase in the area under the curve (AUC) of cortisol over time (8026 ± 1421nmol.min.ml^{-1} vs 1115 ± 881nmol.min.ml^{-1}; p=0.0037). The plasma cortisol levels tended to decrease under the influence of the immobilization when only saline was administered. The increase in plasma cortisol seen in animal B, 20 minutes after Synacthen® administration, arose when the animal stood up unexpectedly. The animal was still blindfolded and remained standing docilely for the remainder of the blood collection period, which was stopped at 70 minutes.

3.2.2 Faecal glucocorticoid metabolites

Figure 3.1 (right panel) shows FGM concentration over time in the four boma-habituated animals (A, B, C and D) in response to administration of Synacthen® or saline. There was no significant difference in the initial value of FGM concentration in the first samples taken directly from the rectum of each animal at the start of each intervention, despite each trial commencing at least seven days apart. All of the animals exhibited an increase in the FGM concentration for the first faecal sample obtained from the enclosure after saline administration. In the control trial, the immobilization of the animals followed by saline administration produced an average FGM concentration of 8.9 ± 2.1ng.g^{-1} over the faecal collection period.

Pharmacologically elevating the plasma cortisol concentration resulted in at least a 3-fold increase in FGM, between 60 and 90 hours later, compared to the baseline value. An average FGM concentration of 14.8 ± 0.7ng.g^{-1} was recorded over this faecal collection period, which was significantly different from when the

saline was injected ($p = 0.0043$). The maximum FGM concentration for each animal following Synacthen® administration was significantly greater than the maximum values recorded following the saline administration ($23.0 \pm 1.2 ng.g^{-1}$ vs $11.7 \pm 2.8 ng.g^{-1}$; $p = 0.0011$). It was noted for animal A and C that the fluctuation observed in the FGM concentration after saline administration was associated with the introduction of new rhinoceroses into adjacent bomas. No cortisol immune-reactivity was detected in the faecal extracts using the ^{125}I cortisol RIA kit.

After the administration of saline, the peak passage of the inert marker occurred in the animals at mean time of 54.1 ± 9.3 hours. After the administration of Synacthen®, the peak passage of the inert marker occurred in the animals at mean time of 76.3 ± 15.8 hours. The increase in gastrointestinal transit time induced by Synacthen® was not quite significantly different ($p = 0.0625$).

Rectal faecal samples obtained from five rhinoceroses captured in the wild had a mean FGM of $4.9 \pm 0.8 ng.g^{-1}$. Fresh faecal samples from these animals obtained from within the boma after the first 5-6 days of captivity had a mean FGM of $11.2 \pm 2.1 ng.g^{-1}$. This significant ($p=0.0005$) elevation in FGM was similar to that observed after Synacthen® administration in the four boma-habituated animals.

Figure 3.2a shows the effect of environmental exposure on the FGM concentration from homogenous samples collected at different time points. When the sample is obtained from the center of each faecal bolus, a delay of up to 240 minutes in obtaining the sample has no significant effect on the FGM concentration obtained (ANOVA; $p=0.8300$)

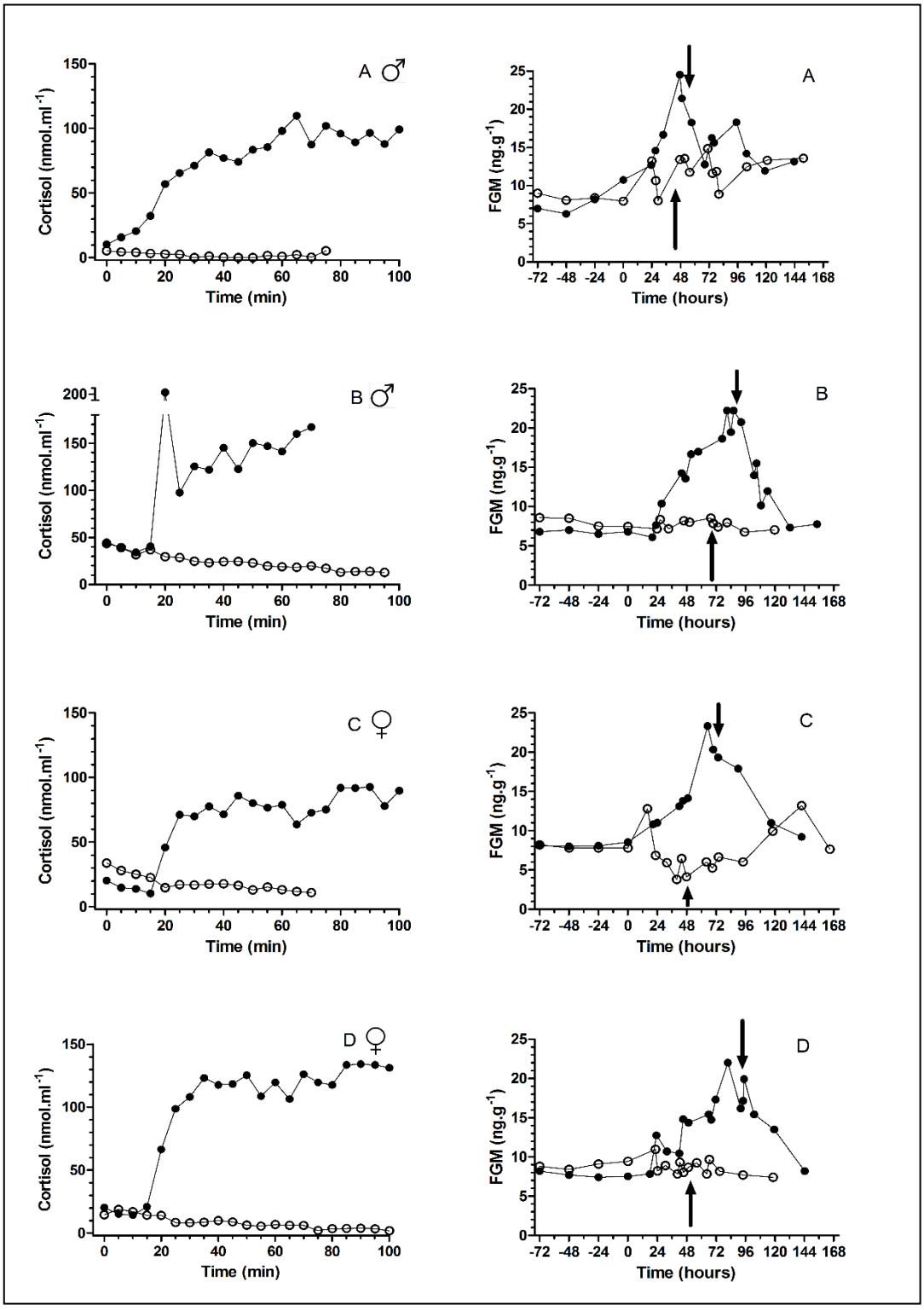


Figure 3.1 Plasma cortisol concentrations (nmol.ml^{-1}) and subsequent faecal glucocorticoid metabolites (FGM; ng.g^{-1} dry faeces) for four boma-habituated white rhinoceroses (A, B, C, D) following an ACTH challenge (•) (1.25-1.5 IU.kg^{-1} Synacthen® i.m.) or saline (o) trial. The arrows (↑) designate the gut transit time for each animal and both trials.

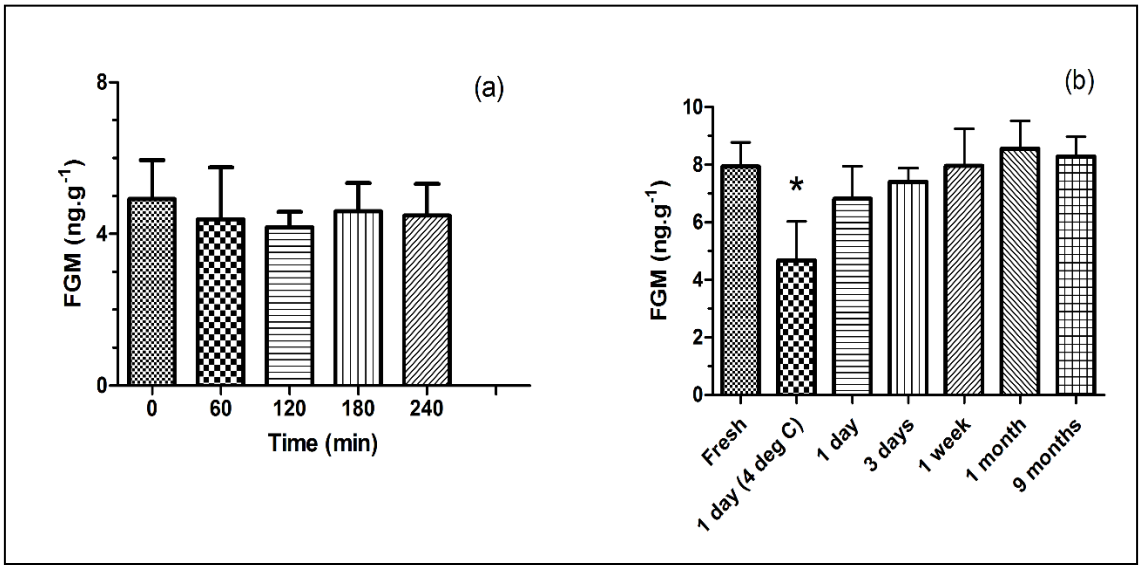


Figure 3.2 Effects of environmental exposure and long-term storage on faecal glucocorticoid metabolite concentrations of white rhinoceros faeces. (a) Environmental exposure (0 to 240 minutes) before collection and freezing, and (b) long-term storage at -20ºC (0 to 9 months) before extraction.
* P< 0.05 different from fresh sample

Figure 3.2b shows the effect of faecal sample storage on the FGM concentration. Storage of the samples at 4°C for 24 hours resulted in the average loss of 41% of the corticoid immune-reactivity in each sample ($p<0.05$). When samples were immediately frozen at <-20°C there was no significant change in corticoid immune-reactivity extracted from each sample for up to nine months, compared to the immediate extraction of a fresh sample.

Table 3.1 gives the data for the within and between assay variation when the RIA assay was used to determine the FGM concentration in the methanol extract. The assay kit produced an acceptable coefficient of variation for a biological assay (4.2%) and this level was maintained between five kits ordered over a two-year period (5.1%). Table 3.2 data shows that the 16-hour rotation of a faecal sample in 80% methanol produced a consistent extraction, while the percent recovery was high and consistent at low (91.7%) and high (89.9%) levels of the spike.

Table 3.1 Within and between assay variation for analysis of the FGM concentration ($ng.ml^{-1}$) in repeated measurements of a single methanol supernatant.

Number of assessments	Mean FGM ± SD ($ng.ml^{-1}$)	Within (%CV)	Between (%CV)
7	0.962 ± 0.040	4.2	----
5	0.925 ± 0.047	----	5.1

Table 3.2 Repeatability and efficiency of the extraction of the FGM from the faecal samples ($ng.g^{-1}$ dry faeces). A single faecal sample was extracted six times, then six aliquots of the same sample were spiked with different amounts of corticosterone before being extracted.

Sample number	Corticosterone added to faeces (ng)	Observed FGM ($ng.g^{-1}$ dry faeces)	%CV	Expected FGM ($ng.g^{-1}$ dry faeces)	% recovery
6	0	11.3 ± 0.61	5.4	----	------
6	5	13.0 ± 0.64	4.9	14.4	91.7
6	20	21.3 ± 0.98	4.6	23.7	89.9

3.3 Discussion

Our data showed that stimulation of the HPA axis through a single intra-muscular injection of Synacthen®, a synthetic analogue of ACTH, produced a significant increase in the plasma cortisol concentration for all the white rhinoceroses compared to a control saline injection. This cortisol increase subsequently induced a significant increase in the FGM concentration of the rhinoceroses. The data from the five animals captured in the wild and then confined in the boma confirms that this physiological/psychological stressor was sufficient to produce a detectable

increase in the FGM concentration, relative to the faecal sample obtained at capture.

The plasma cortisol concentration that was achieved in white rhinoceroses with this dose of Synacthen® resembles the response to acute stress caused by the capture and immobilization processes (Kock et al., 1990). The average plasma cortisol concentration obtained in this study was also representative of the plasma cortisol concentrations we have measured in rhinoceroses after capture, confinement and translocation (see Chapter 4). Faecal cortisol could not be detected with the Coat-a-Count RIA kit, a finding that has been confirmed by other investigators (Brown et al., 2001) but faecal cortisol is evidently detectable using HPLC (Turner et al., 2002). Due to the small sample size of our study no comparisons could be drawn between animals of different ages and sexes.

The FGM concentrations of the faecal sample obtained at the start of each intervention are similar to faecal corticosterone concentrations measured by HPLC in four captivity-habituated white rhinoceroses (range 4.0-10.8 $ng.g^{-1}$ over 21 days) (Turner et al., 2002). The maximum increases in FGM concentration measured by this RIA method after Synacthen® administration are also comparable to the 3-to 4-fold increase in faecal corticosterone concentration obtained from rhinoceroses following capture, confinement and translocation (Turner et al., 2002). This significant adrenal response to the ACTH challenge is evidence that boma-confinement for 90 days is not associated with adrenal gland fatigue. Declining FGM concentrations with time in captivity are normally taken to indicate adaptation to the stressors of confinement (Turner et al., 2002). However, the association of reduced FGM concentrations over time together with reduced faecal androgen or progestin levels has been linked to distress with adrenal malfunction in rhinoceroses (Linklater et al., 2010). Our FGM data following an ACTH challenge clearly show that this cannot be the reason. Chronic social stress could also not be linked to reduced reproduction in another rhinoceros study (Metrione and Harder, 2011). The only association of lower than normal FGM concentrations with a biological cost has been that of compromised

immune function in the black rhinoceros (Dorsey et al., 2010) and further investigation is warranted.

Baseline FGM concentrations in our study are significantly lower than those found in white rhinoceroses confined in zoos (Brown et al., 2001; Carlstead and Brown, 2005). Even though the same corticosterone RIA kit was used differences in the faecal extraction method and perhaps diet may account for this observation. I believe that our study has adequately quantified the performance of the assay in terms of its suitability, consistency and precision, which was not available for most other studies. One possible consideration is that other investigators may have failed to take cognisance that the concentration of the corticosterone standards in the kit have been adjusted to take account of a dilution step (see Methods above). However, some preliminary data from rhinoceroses that have been boma-confined in the KNP for up to two years suggest that significant up-regulation of the HPA axis can be expected, which may have occurred in the zoo animals. The use of wild-captured, boma-habituated white rhinoceroses probably gives the clearest picture of physiological cortisol and FGM dynamics as these animals are only kept in captivity for relatively short periods of time. Furthermore, young healthy animals are normally selected for translocation to establish new breeding groups.

Our study also highlights the effect of stress on the gastrointestinal transit time. The mean gastrointestinal transit time determined for the white rhinoceroses following saline injection was 54 hours. Following the ACTH challenge the peak clearance time was increased on average by 20 hours. Since the administration of either the saline or ACTH was randomized, this increased clearance time cannot be attributed to the experimental protocol. The implication is that under conditions of increased blood cortisol concentration it can be expected that gastrointestinal transit time is extended and may be associated with reduced feed intake. This finding may be a significant factor contributing to the anorexia observed in some rhinoceroses immediately after capture at the start of the confinement period. In addition, this extended gastrointestinal transit time should be considered when evaluating stressed rhinoceroses to ensure that the measurement of FGM corresponds with the timing of the stressor being evaluated.

ACTH challenge tests have been reported for four male black rhinoceroses (Brown et al., 2001), a single black rhinoceros male (Santymire et al., 2012), a single male Indian rhinoceros (Capiro et al., 2014) and a single wild-capture female white rhinoceros (Metrione and Harder, 2011). None of these animals were anaesthetized for blood collection, but blood samples were obtained for corticoid determinations from two black rhinoceroses (Brown et al., 2001) and the female white rhinoceros (Metrione and Harder, 2011). FGM concentration following ACTH challenge was not determined for the white rhinoceros and only a 20-fold increase in serum corticosterone was reported. Blood cortisol increased by 10 and 16-fold in the two male black rhinoceroses while a peak in excreted FGM following ACTH occurred at about 48 hours (Brown et al., 2001) and 75 hours (Santymire et al., 2012). For the Indian rhinoceros a 3.5-fold increase in FGM was recorded with the peak excretion at about 22 hours following ACTH injection (Capiro et al., 2014). In the current study the rigorous experimental design allowed more information to be gained. Firstly, the saline control condition confirmed that high blood cortisol following the ACTH challenge was associated with a prolonged gastrointestinal transit time. Secondly, the saline control condition confirmed that cortisol secretion decreases under anaesthesia up to 90 minutes. Finally, the control condition confirmed that even the stress of chemical immobilization can be detected by an increase in FGM at about 24 hours after the event, which is earlier than it takes for the orally administered marker to exhibit a peak in the faeces. This fact suggests that a small amount of the plasma cortisol may be transferred across the wall of the gastrointestinal tract and be metabolized in the gastrointestinal tract, rather than be excreted via the bile. Supporting evidence for this assumption comes from an ACTH challenge in lactating cows (Termeulen et al., 1981) where cortisol appearance in milk mirrored that in the blood. This means that even a small elevation of blood cortisol in the rhinoceroses in response to the capture process, or following the anaesthetic reversal, may have had a rapid effect on faecal FGM levels, as seen in the first faecal samples collected in our study. Samples collected from the rectum at capture should thus be interpreted with caution, since they may not reflect baseline FGM levels if the capture was particularly stressful or prolonged.

The extraction and assay procedures adopted have considered the methodological considerations raised by experienced researchers in this field (Millspaugh and Washburn, 2004; Palme, 2005). Faecal samples were well-mixed and a portion of the sample was dried to establish the percentage water content. This step is important because the consistency of rhinoceros faeces varies over time during habituation of the animals to the boma. In addition, no significant difference in the FGM concentration was found between fresh samples collected from the rectum and samples that had been effectively collected from the boma floor up to four hours later. Similarly, the faecal samples can also be stored at <-20ºC for up to nine months before being extracted. Directly assaying the methanolic extract in a steroid diluent was similar to the procedure adopted by other researchers (Dorsey et al., 2010; Termeulen et al., 1981) and gives excellent sample recoveries as well as a respectable coefficient of variation between samples in our assay (Dorsey et al., 2010; Terio et al., 1999). All of these factors make for a robust and reliable assessment of FGM concentrations in the rhinoceros.

Although this FGM measurement is non-invasive, relevant and robust, the time required to complete the extraction and assay could take several days. This data can be advantageous to monitor stress of animals confined to bomas, but has no application where animals are captured and immediately translocated to other wild areas. It would therefore be advantageous to be able to predict the fate of an animal based on measurements made at the time of capture, which requires a more rapid field assessment to be made on site. To achieve this goal, we continued to evaluate the role that FGM information can provide in validating additional measures of animal stress and likely post-translocation outcomes.

CHAPTER 4

Hypothalamic-Pituitary-Adrenal axis responses to capture and boma confinement in the white rhinoceros

A large number of white rhinoceroses are captured and translocated every year in the Kruger National Park, South Africa. This is done to contribute to the long-term survival of the species and to diminish the impact of poaching (Armstrong and Seddon, 2008; Dickens et al., 2010; Ferreira et al., 2012; Pinter-Wollman et al., 2009). In the wild the rhinoceroses are darted from a helicopter (Miller et al., 2013) and are either transported directly to a new destination or placed in holding facilities called bomas (an enclosure to hold live wild animals) in preparation for their subsequent translocation. This temporary confinement is commonly referred to as boma-training.

Boma-training involves a process of habituating or desensitizing animals to the stressors of confinement (Cyr and Romero, 2009; Grissom and Bhatnagar, 2009). As part of this training, they are also gradually introduced to different types of hays. The intensive management of the wild-captured rhinoceroses is essential to ensure successful habituation, reduce injuries, and avoid mortalities. The habituation process involves gradually introducing the rhinoceroses to incrementally smaller areas within the bomas (Figure 2.1; see Chapter 2) at a rate determined by each individual rhinoceros's behaviour. By applying moderate stressors to an animal for a short period of time they habituate and the magnitude of their response to the stressor decreases (Esteruelas et al., 2015; Grissom and Bhatnagar, 2009; Råberg et al., 1998; Romero et al., 2009).

After capture, the rhinoceroses are placed in a large "introduction boma" of the complex for 7 to 14 days. The time spent in the introduction boma is determined by how well the rhinoceroses habituate to their confinement. This is subjectively determined by the amount of food the rhinoceroses eat, subsequent amounts of defecation, and their behavioural responses to the confinement conditions. Once

the rhinoceroses are eating and defecating well, they are then placed in a smaller boma, the “confinement boma”, which has two compartments for management purposes. Each rhinoceros will stay in this smaller area for at least 14 to 28 days before its translocation can be considered. During this time period they may be moved to other more appropriate bomas, depending on the dominance hierarchy of neighbouring rhinoceroses (McEwen and Seeman, 1999; Metrione et al., 2007).

In the wild, formation of dominance relationships provides the basis for competition for essential resources. White rhinoceroses in the wild have a social system that is based on clearly defined adult male territories. This territorial behaviour is characterized by four features; range exclusiveness, ritualized encounters, confinement of oestrous cows and scent marking (Owen-Smith, 1971). In captivity, dominance hierarchies also develop as a result of herd social structure and competition for resources in white rhinoceroses, this hierarchy should always be considered when animals are placed in captivity to avoid spatial and social stress in the animals (Metrione et al., 2007).

Stressors suppress feeding behaviour in some animals (Sapolsky et al., 2000) and this phenomenon is also noted in the rhinoceros. Those rhinoceroses that fail to eat or defecate sufficiently within the first 10 to 14 days of confinement are released back into the wild before any further serious health-related problems occur. Opportunistic infections, such as *salmonella* have also previously been noted in animals that fail to eat and adapt in the bomas.

Stressors such as immobilization, handling, confinement and novel environments result in elevated blood concentration of the glucocorticoid hormone, cortisol, in animals (Dickens et al., 2010; Kock et al., 1990; Kock et al., 1999; Linklater et al., 2010; Merl et al., 2000). This increased concentration of plasma cortisol typically returns to physiologically normal concentrations in time, resulting in habituation (de Kloet et al., 2005; Franceschini et al., 2008; Hill and Broom, 2009; Romero, 2004; Turner et al., 2002). Habituation can also be described as the process that occurs when the same stressor(s) is applied to animals over a prolonged period of

time (Dhabhar and McEwen, 1997). Habituation involves the negative feedback to the brain by cortisol to reduce the secretion of corticotrophin-releasing-factor (CRF) and adrenocorticotropic hormone (ACTH). If there is either a sustained activation of the hypothalamus or a deficit in the efficiency of the negative feedback, elevated plasma cortisol concentrations can occur for a longer period, resulting in negative effects on the animal, such as lack of habituation (Romero, 2004; Romero, 2012; Romero and Wikelski, 2010). It has been proposed that chronic stress and/or lack of habituation has negative effects on numerous physiological systems, including reproduction, growth, metabolism, immune function, behaviour and subordinate status by stimulating endocrine responses (Berga, 2008; Breazile, 1987; Budzyńska, 2014; de Kloet, 2003; Harbuz and Lightman, 1992; Morley et al., 1991; Powell et al., 1967b; Sapolsky, 2010; Stratakis and Chrousos, 1995; Wikgren et al., 2012; Wingfield and Sapolsky, 2003). Human and laboratory animal studies have indicated that chronically elevated concentrations of the stress hormone cortisol are also associated with anorexia and depression (Sapolsky et al., 2000; Brambilla, 2001).

There are few similar studies in the literature, but a study of dogs entering kennels showed they had an increase in hypothalamic-pituitary-adrenal axis (HPA) activity, but the increase was significantly higher in non-habituated dogs (Rooney et al., 2007). The HPA activity in non-habituated dogs also stayed above baseline values for longer than in dogs that had previously been partly habituated (Rooney et al., 2007). Rats that were repeatedly handled also showed a decrease in the HPA response, which was paralleled by a reduced behavioural response, when compared with rats handled only on a single occasion (Dobrakovová et al., 1993). In a further study exposure of rats to a severe stressor, in this case physical immobilization, caused an increased HPA response and an increase in anxiety-like behaviour when the rats were placed in a maze after exposure to the stressor (Armstrong and Seddon, 2008). A single exposure to a stressor is not only capable of inducing a long-lasting HPA response, it can also induce a behavioural and physiological sensitization of the response to further stressors (Belda et al., 2008).

Animals cope with changing environments by using both behavioural and physiological stress responses (Budzyńska, 2014). In the wild-captured rhinoceroses, the translocation procedure causes a stress response which has both a behavioural component and an adaptive physiological component. The stressor activates the immediate fight-or-flight response of the sympathetic nervous system, which is followed by the longer hormonal stress response of the HPA axis (Sapolsky et al., 2000). In nature, where a stressor mostly diminishes or ceases after a short period of time, negative feedback quickly suppresses the cortisol release, this is described as an acute stress response (Dickens et al., 2010). However, when the rhinoceroses are moved directly from the wild into confinement this causes a series of acute stressors on the rhinoceroses, resulting in a condition of chronic stress which can lead to the non-adaptation mentioned above (Wingfield and Romero, 2010). There is a bidirectional interaction between hormones and behaviour, where hormones can affect behavioural response and behaviour can influence hormone concentrations (Budzyńska, 2014). Chronic stress in an animal can cause changes in the animal's physiological responses (HPA axis function and related effects) and behavioural responses (Dickens et al., 2010).

The objective in this study was to establish whether there is any evidence that the wild rhinoceroses habituate to confinement. We hypothesized that rhinoceroses that show early habituation to the confinement do so because there is reduced activation of the HPA axis. This study further investigated whether the magnitude of initial activation of the HPA response at capture contributes to the long-term HPA response observed during confinement. If this were true, then measures of samples taken at capture could be used to predict the outcome in the bomas. In addition, we used an established method (Miller et al., 2016) of scoring rhinoceros behaviour in confinement, to determine if the observed behaviour correlated with HPA activity recorded during confinement.

4.1 Methods

To determine the extent of HPA axis activation in response to the initial stress of capture, handling and transportation, blood samples were obtained from each rhinoceros. To assess the effect of confinement on HPA activity, faecal samples were obtained at capture and at every opportunity during the period of confinement for the measurement of FGM as described in Chapter 3.

4.1.1 Animal subjects

This study was conducted in the Kruger National Park (KNP), South Africa, on a wild population of white rhinoceroses (N = 8968) (Ferreira et al., 2015). Some of these animals were authorized for translocation within South Africa during 2008 to 2012 (n = 107). The rhinoceroses were immobilized from a helicopter as described in Chapter 2.

4.1.2 Data collection at capture

To determine which variables, if any, were associated with the subsequent responses to confinement the following relevant data were recorded: season, sex, ambient temperature at capture, distance the rhinoceros travelled before darting and after darting, body temperature once recumbent, time spent in crate before release into the boma and body weight at capture.

4.1.3 Sample collection

4.1.3.1 HPA response to capture

To determine what the HPA response of each rhinoceros was to all the procedures of the capture operation, blood samples were collected in the wild from each immobilized rhinoceros immediately when it became recumbent. The blood samples were collected from the auricular vein into evacuated tubes (BD

Vacutainer systems, Preanalytical Solutions, Plymouth, UK). A further blood sample was obtained immediately before the rhinoceros was released into the boma (30 to 90 minutes after capture; time spent in the transport crate). In the laboratory, plasma and serum were harvested by centrifugation, and aliquots were stored at -20°C until assayed.

4.1.3.2 HPA response to confinement

To determine what the HPA response of the rhinoceros was to boma-confinement, a fresh faecal sample was collected from the rectum of each rhinoceros at capture. Further samples were then attempted to be collected from faecal piles in the boma of each rhinoceros in the morning between 7:00 and 8:00 and again whenever else there was an opportunity to do so, up to day 28 after capture. After the period of boma-habituation each rhinoceros was partially immobilized for translocation to its final destination. At this time, a rectal faecal sample and a blood sample were again obtained. All faecal samples were mixed well and approximately 100g of each was frozen at -20°C until FGM extractions were performed. Plasma was harvested by centrifugation, and aliquots were stored at -20°C until the assay was performed.

4.1.3.3 Behavioural response to confinement

All rhinoceroses which entered the boma were carefully monitored and their well-being scored on a daily basis according to a simple three-category scoring system. This validated scoring system is based on appetite, faecal consistency and volume, and behaviour (Miller et al., 2016). Each category contributes a maximum of 5 points to the total score; a high score represents a rhinoceros that habituated well to confinement: eating well, defecating well and not aggressive or nervous. A low score, on the other hand, represents a rhinoceros that did not eat or defecate well and appeared to be aggressive, restless or nervous (Table 4.1). All the activities

Table 4.1 Scoring system for the monitoring of white rhinoceros (adapted from Miller et al. 2016).

	Score	Appetite	Faecal consistency/volume	Behaviour
HEALTHY ANIMAL	5	Eating 75% to 100% of normal intake[a]	Brownish/green large stool (multiple defaecations per day)	Calm and alert, but doesn't avoid people; standing stationary and turns head and/or ears towards stimulus[b]
	4	Eating 50% to 75% of normal intake[a]	Dark brownish/green medium stool (3 to 5 balls more than once a day)	Calm but avoids people; walks away slowly in response to stimulus[b]
	3	Eating 25% to 50% of normal intake[a]	Dark small stool (1 or 2 balls more than once a day)	Mildly nervous and/or aggressive; trots and/or walks away rapidly for a short distance in response to stimulus[b]
	2	Eating 0% to 25% of normal intake[a]	Putty-like dark, small stool or loose faeces	Moderately nervous and/or aggressive; runs or trots away and/or charges once or twice in response to stimulus[b]
	1	Not eating at all	Not defaecating	Extremely nervous and/or aggressive; runs around and/or frequently charges and/or hits the poles/doors in response to stimulus[b]
SICK / ILL ANIMAL	-1	-	Stool is loosely formed (similar to domestic cow)	Mildly depressed; minimal movement of ears, partially closed eyes, walks slowly only short distance in response to stimulus[b]
	-3	-	Diarrhea (light brown or green in colour)	Moderately depressed; no response of ears, eyes, or movement while standing in the presence of stimulus[b]
	-5	-	Profuse watery diarrhea (dark brown / black in colour)	Very depressed; recumbent with no response to stimulus or physical touch[b]

[a] Normal intake for an adult white rhinoceros is approximately 25–40 kg of mixed a per day. Animal size and type of feed will affect the amount that is considered normal intake and should be adjusted for each facility based on consumption by adapted animals.

[b] Stimulus = presence of a person at ground level in view of the animal close to the poles of the boma.

related to the management of the rhinoceroses while in confinement were also recorded; this included time spent in the introduction boma, when the rhinoceros was closed in the confinement boma, whether the rhinoceros was moved to an alternative confinement boma and information related to neighbouring rhinoceroses (age and sex). Finally, the weight of the rhinoceros was recorded when it was loaded for translocation.

4.1.3.4 Plasma cortisol assay

Plasma cortisol concentrations were measured using a commercially available ^{125}I RIA kit (Coat-a-Count®, Siemens Healthcare Diagnostics, CA, USA).

4.1.3.5 FGM extraction and assay

Extraction of glucocorticoid metabolites from the faecal sample was done using the method described in Chapter 3. The measurement of FGM concentrations was performed with a ^{125}I Corticosterone RIA kit (MP Biomedicals LLC, Santa Ana CA, USA) which has been validated for several species including South African herbivores (Brown et al., 2001; Carlstead and Brown, 2005; Chinnadurai et al., 2009; Wasser et al., 2000). The methanol extracts were diluted 2:1 with the manufacturer's steroid diluent (Cat. No. 07-166196) and assayed according to the manufacturer's instructions. Results were recorded as ng.g^{-1} dry faeces.

4.1.3.6 Data analysis

Plasma cortisol concentrations were not normally distributed so they were compared by repeated measures, non-parametric Friedman test and Dunn's multiple comparison post-test. Gender differences were tested for with the use of the Mann Whitney test. FGM data were also not normally distributed so values obtained on different days were compared with the Kruskal-Wallis statistic and Dunn's multiple comparison post-test. To test whether the HPA response at capture influenced the HPA response in confinement, the Spearman correlation

coefficient was determined using plasma cortisol concentrations at capture and FGM concentrations during confinement. To establish whether any of the other variables associated with the capture process had an effect on the HPA response, the Spearman correlation coefficient was used to examine their association to the plasma cortisol concentrations and the subsequent FGM concentrations measured. Each rhinoceros's individual scoring was also compared against the capture variables for any association by determining the Spearman correlation coefficient.

4.2 Results

4.2.1 HPA response to capture

There was no difference in the plasma cortisol concentrations between male and female rhinoceroses at any point, so data for both sexes were combined. Figure 4.1 shows the data obtained for plasma cortisol concentrations of white rhinoceroses at the time of capture, when the rhinoceroses were offloaded at the boma (after spending time in crate) after capture in the wild and when the rhinoceroses were immobilized and reloaded for translocation. Blood sample were collected from 124 animals at capture but figure 4.1 shows the results of 107 complete datasets that were available.

By the time the rhinoceroses were offloaded at the boma the plasma cortisol was significantly ($P<0.05$) higher than from the sample obtained at capture ($103.6 \pm 43.4 nmol.l^{-1}$ vs. $43.1 \pm 26.1 nmol.l^{-1}$; n=107). After the period of confinement, the plasma cortisol concentration was similar to that obtained at capture ($36.8 \pm 24.1 nmol.l^{-1}$vs. $43.1 \pm 26.1 nmol.l^{-1}$; n=107), but was significantly ($P<0.05$) lower than when the rhinoceroses were released into the boma ($36.8 \pm 24.1 nmol.l^{-1}$ vs. $103.6 \pm 43.4 nmol.l^{-1}$; n=107).

The plasma cortisol concentration at capture did not significantly correlate with the plasma cortisol concentration obtained when the animals were offloaded at the

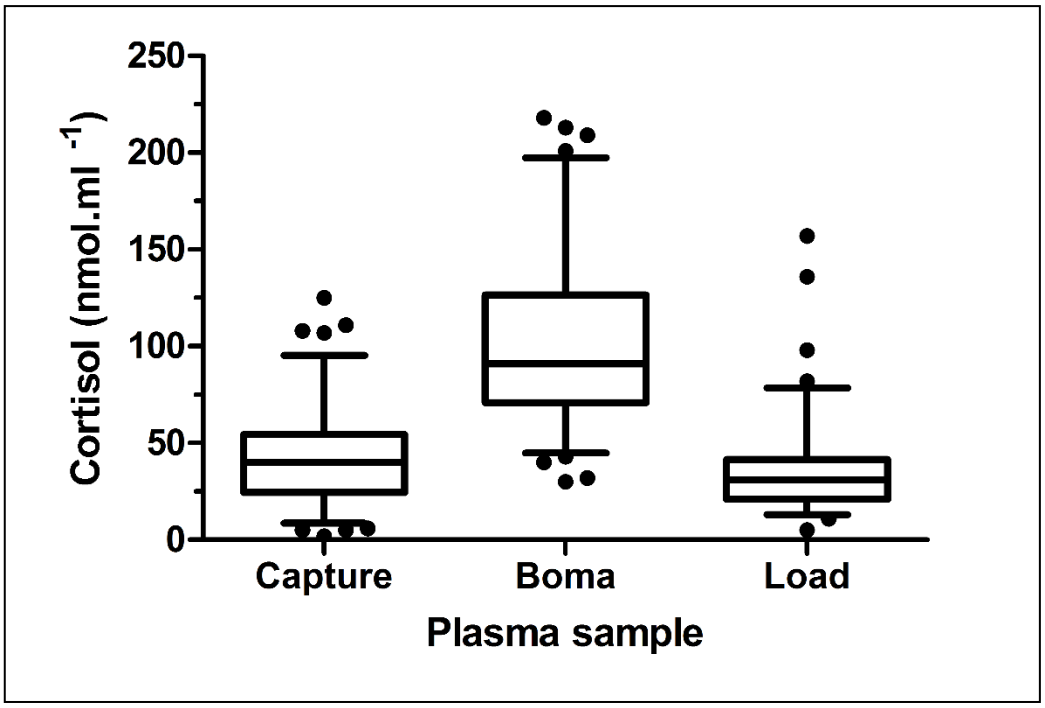


Figure 4.1 Plasma cortisol concentrations (nmol.l^{-1}) of white rhinoceroses (n=107) obtained at capture, before offloading at the boma and later when reloaded for translocation Data is shown as box plots with median and 95% confidence levels.

boma ($S_p r$ = 0.1637; P=0.0753; n=107). The results in Table 4.2 show that neither age of the rhinoceros nor season of the year showed any correlation with the plasma cortisol concentration at capture or when the rhinoceroses were offloaded at the boma.

The distances the rhinoceroses ran before darting, after darting and therefore the total distances run have an influence on the plasma cortisol concentration at capture. However, by the time the rhinoceroses were offloaded at the boma there was no significant correlation between these variables and plasma cortisol concentration. The distance the animal moved before being darted as well as the season in which the capture took place also had an influence on the body temperature.

4.2.2 HPA response to confinement

Figure 4.2 shows the trend of the FGM concentration excretion from the time of capture (rectal sample) over the first 28 days of confinement (boma floor samples) and again at the time of loading for translocation (rectal sample). The FGM concentrations obtained from the samples of the rhinoceroses while they were confined in the boma were all greater ($P<0.05$) than from the samples obtained from the rectum of the rhinoceroses at capture (4.2 $\pm$ 1.6ng/g; n=59). There was no significant difference in FGM concentration from the samples collected in the boma between day 7 (9.0 $\pm$ 5.0ng/g; n=19), day 14 (9.2 $\pm$ 5.5ng/g; n=40), day 28 (7.6 $\pm$ 4.0ng/g; n=48) or when the rhinoceroses were loaded for translocation (6.6 $\pm$ 2.5ng/g; n=54).

No significant difference was found in the FGM profiles in the females compared to the males over 28 days, except between day 7 and day 10. From day 7 to 10 the

Table 4.2 Capture conditions which influence plasma cortisol concentrations and body temperature.

Variable	Plasma cortisol concentration at capture		Plasma cortisol concentration at offloading		Body temperature	
	S_pr	P	S_pr	P	S_pr	P
Distance before darting	0.3642	0.0071	0.2331	0.1109	0.3699	0.0205
Distance after darting	0.4121	0.0029	0.2623	0.0782	0.1741	0.2958
Total distance	0.4020	0.0032	0.2737	0.598	0.2981	0.0653
Age	-0.144	0.344	0.054	0.701	-0.0014	0.9929
Season	-0.0460	0.7296	-0.0708	0.6073	-0.5817	0.0000

Spr = Spearman correlation coefficient; P = probability

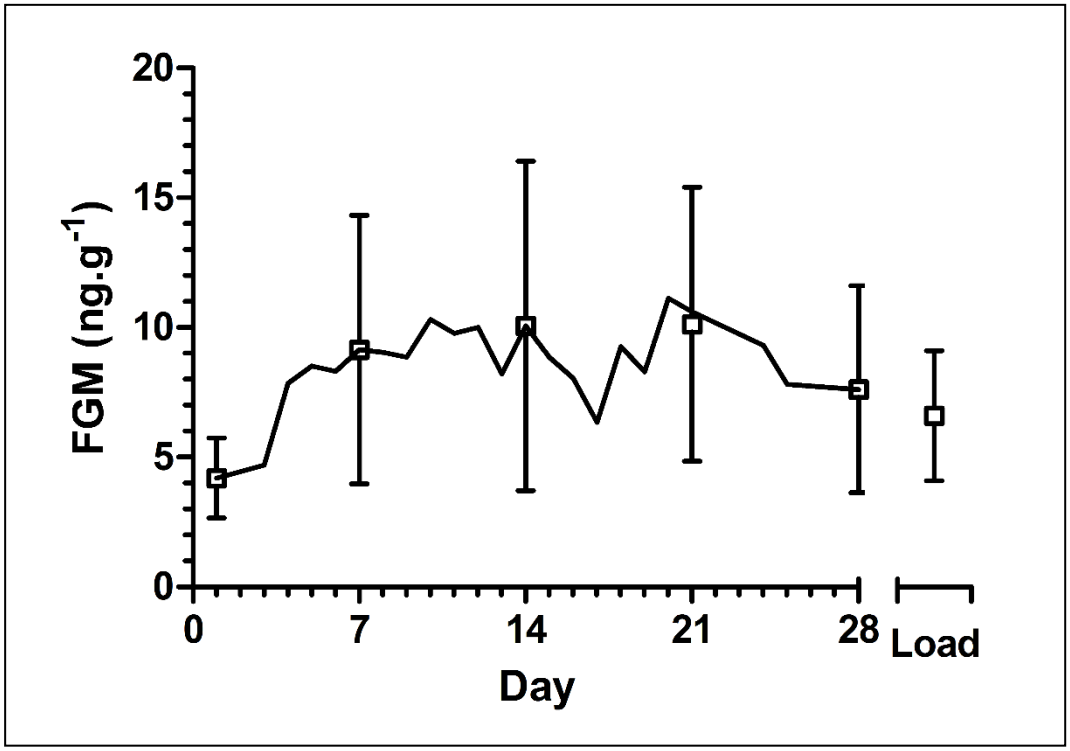


Figure 4.2 Faecal glucocorticoid metabolites ($ng.g^{-1}$) of white rhinoceroses measured in faecal samples obtained for up to 28 days of confinement. For clarity, the average data are represented as a line showing the average data over the 28-day period, with mean and standard deviation only shown for day of capture and then on days 7, 14, 21 and 28.

4.2.3 Behavioural response to confinement

The average behavioural score for individual rhinoceroses in confinement did not correlate with FGM concentrations, plasma cortisol concentrations at capture or any of the other capture variables. The 7-10 day FGM concentration was, however, negatively correlated with individual behavioural scores between day 7 and 10 (S_pr = -0.4453; P = 0.021; n=31). Plasma cortisol concentration in the blood sample collected at capture was also negatively correlated with behavioural scores between day 7 and 10 (S_pr = -0.3860; P = 0.0351; n=30).

4.3 Discussion

In this study, we found that white rhinoceroses have variable responses to capture. The rhinoceroses showed a significantly increased cortisol secretion in response to transportation after capture, which did not correlate with the time spent in the crate before being offloaded at the boma or with the plasma cortisol concentration at capture. This suggests that there is a differing individual response to handling and transportation after capture.

Similar to the ACTH validation study (see Chapter 3), rectal faecal samples obtained from wild-captured white rhinoceroses show low concentrations of FGM. However, within 4 days of confinement after capture there was a significant increase in FGM concentration which was associated with strong stimulation of the HPA axis. It is important to note that during the initial three days of confinement the rhinoceroses are still under the sedative effect of the Acuphase which was injected at capture (see methods). Declining FGM concentrations are observed from day 14 onwards to day 17, but have a tendency to increase in the five days thereafter. It was at this time that the rhinoceroses are often moved within the boma complex for better placement and management in the boma. The only difference noticed in different sexes of white rhinoceroses in response to confinement was that the females' FGM concentrations peak earlier than the males'. A similar response was seen in Southern American camelids where there were also no significant differences in FGM concentrations in the different sexes but it also peaked at different days and at different concentrations (Arias et al., 2013). The female rhinoceroses showed better behavioural habituation to boma confinement than the male rhinoceroses and could be enclosed in the confinement boma as early as day 7 to 10. For the males, confinement to the smaller bomas could only take place between day 10 and 14. The higher FGM concentration recorded in the females compared to the males at this time (day 7 to 10) suggests that the HPA axis was once again stimulated by this confinement in the smaller boma area.

It is important to note that even for an extended period of confinement, when a rhinoceros is considered to be well habituated, the FGM concentration does not return to pre-capture levels. Despite the fact that the individual scores (feeding, defecation and behaviour) of the rhinoceroses in confinement improved over time, the elevated FGM concentrations suggests that there is still significant cortisol secretion. After 7 to 10 days of confinement, the increasing FGM concentrations and lower scores suggested greater perceived stress by the rhinoceroses. The only valid predictor of rhinoceroses displaying these behaviours was the HPA reactivity as indicated by the elevated plasma cortisol concentrations at capture. Although there was evidence of behavioural habituation, the HPA axis activity shows that there was no physiological habituation.
In our study, the majority of the rhinoceroses responded in a similar way to that previously found in black rhinoceroses (Kock et al., 1990) and black and white rhinoceroses (Turner et al., 2002). The study on black rhinoceroses (Kock et al., 1990) showed an increase in plasma cortisol concentration with capture and confinement, while another study on black and white rhinoceroses (Turner et al., 2002) showed an increase in FGM concentration with capture and confinement.

It is crucial for the cortisol secretion to be shut off via negative feedback after an acute stressor diminishes or ceases, as elevated cortisol concentrations can potentially cause physiological damage (Romero and Wikelski, 2010). Some of the variables involved with the translocation process of rhinoceroses cause unavoidable stress to the rhinoceroses and have the potential to cause chronic stress. The translocation process involves multiple acute stressors that initiate consecutive acute stress responses as well as longer lasting chronic stress responses. Chronic stress can lead to changes in both the physiological stress response and the behavioural response; this includes HPA dysregulation and behavioural coping. These responses themselves or in combination with others (increased sympathetic nervous system output and immune suppression) contribute to translocation failure because they are tied to factors directly linked to translocation failure such as starvation, increased susceptibility to disease, reproductive failure, predation and dispersal (Dickens et al., 2010).

It is important to minimize the accumulated multiple acute stressor responses through proper planning and management. Effective planning would include making use of the best available equipment, technology, pharmacology and highly experienced personnel throughout the entire translocation operation. Design of the holding facility with large areas for the introduction boma is very important for success. Intensive management of the rhinoceroses in confinement requires providing the best available fodder and water, minimal disturbance of the rhinoceroses and limiting unnecessary moving of rhinoceroses in the complex. Non-invasively monitoring the HPA response of the rhinoceroses to confinement also gives insight that allows management changes to be implemented. By evaluating FGM concentrations of rhinoceroses throughout the boma period, changes in the HPA axis response could be controlled by management practices.

Evidence for the above recommendations can be noted by looking specifically at typical individual responses of male and female rhinoceroses in confinement as shown in figure 4.3 and discussion below.

Male 39/09 (five to six years old). This rhinoceros started eating at day 3 but was always slightly nervous. He was closed in the confinement boma at day six and moved to another boma the next day. This was followed by the first spike in the faecal glucocorticoid metabolite (FGM) concentration. The FGM concentration then decreased to day 9 when a neighbouring animal was immobilized for loading and translocation. This activity resulted in another small spike at day 11. On day 14 a larger male rhinoceros was moved into the boma next to him, resulting in another spike in the FGM concentration. After this HPA axis response this animal seemed to settle down and the FGM concentration on day 28 was slightly greater than the pre-capture level.

Male 34/09 (10 to 15 years old). This rhinoceros had been captured four years previously and placed in the boma. He, however, did not eat well after the first capture and had been released back into the wild at day 10. This time around he was nervous and aggressive for the first three days but started eating by day 4,

which was followed by a decrease in the FGM concentration. Introducing other rhinoceroses in the adjoining bomas caused a small spike toward day 9 and 10. On day 14 he was closed in the confinement boma which caused the biggest FGM concentration increase. The FGM concentration then decreased by day 28, but did not reach the pre-capture level.

Male 40/08: (six to seven years old). This rhinoceros had recent scars on his head from fighting in the wild. Scars like these are normally obtained from fights with territorial males. Of particular note is the elevated FGM measured in the rectal sample obtained at capture. He also fought with the young female that was captured at the same location with him and who was released into the same boma on arrival. They were immediately separated and placed in individual bomas. By day 2 he started eating and this improved on day 3 and 4. On day 6 he was moved to another boma where his neighbours were other larger males. This management action caused the start of a spike in FGM concentration from day 8 and to day 14 when it peaked. He was subsequently closed in the confinement boma, which isolated him from the other males on day 14 which resulted in a small decrease in the FGM concentration for the next three days. FGM concentration however had increased by day 28. This case shows that despite the fact the rhinoceros was eating well it never habituated to the confinement, as seen in the elevated FGM concentration.

Female 8/09 (two to three years old). This animal was nervous and aggressive from the first day she entered the boma, but she was eating from day 3. Her FGM concentration peaked at day 5, which coincided with the appearance of several superficial wounds which she had sustained in the boma, by charging the pole walls and gates. She subsequently became less nervous and aggressive and was closed in the smaller confinement boma on day 8. This action was associated with a small spike in the FGM concentration by day 10. She remained nervous and aggressive and had settled down somewhat in time, but by day 28 the FGM concentration was still elevated.

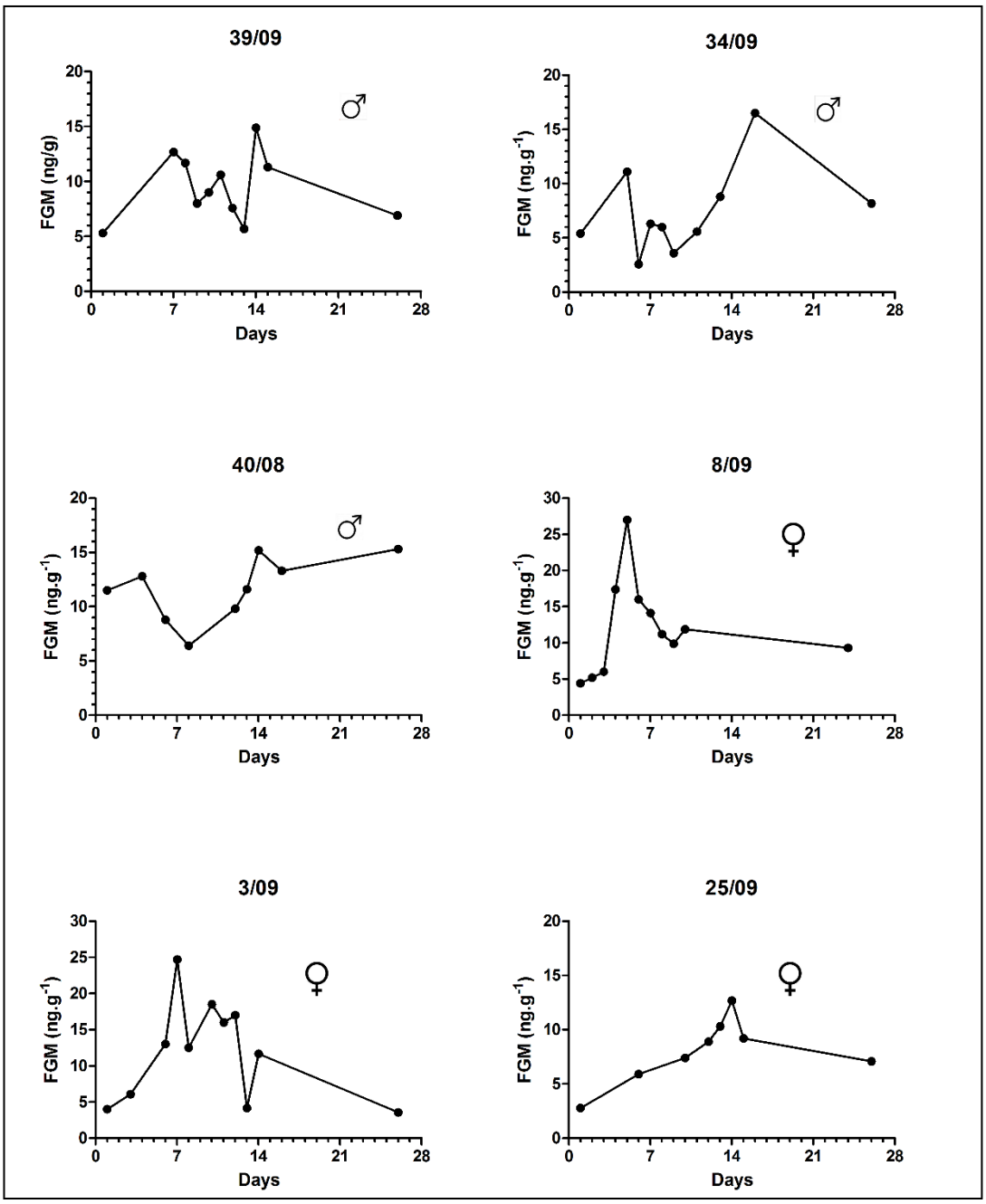


Figure 4.3 Individual responses of male and female white rhinoceroses to confinement and management actions.

Female 3/09 (six to seven years old). This animal was also nervous and aggressive when she entered the boma. She started eating the fresh grass in the introduction boma by day 3 but paced up and down the boma. The FGM

concentration peaked by day 7 but she seemed to have settled down. By day 8 and 9 she however started hitting the poles and digging holes, which caused a small spike in FGM concentration by day 10. She was closed in the confinement boma on day 11, when she also settled down. The FGM concentration returned to pre-capture level by day 28.

Female 25/09 (six to seven years old). This animal was aggressive from the time she entered the boma. On day 2 she was digging holes and showed aggression resulting in her chipping her front horn. On day 3 she was hitting the boma doors and it was decided to dart her with a drop-out dart containing zuclopenthixol acetate (Acuphase®, H. Lundbeck A/S, Ottiliavej 9, DK – 2500, Valby, Denmark). This had the desired behavioural effect as she was able to be closed in the confinement boma on day 5. The FGM concentration also increased at a slower rate than the other females, most likely due to the effects of the tranquiliser. By day 8 she was eating well but became nervous and aggressive again. On day 12 she was moved to another confinement boma and she split her front horn the next day. FGM concentrations reached a peak by day 14. She eventually broke the horn off by day 22, but seemed to have settled down thereafter. The FGM concentrations then declined but never reached pre-capture level.

These case studies presented above indicate that FGM concentrations closely reflect HPA activity in response to both animal interpersonal conflicts and human management practices within the bomas.

4.4 Conclusion

In this study the capture, handling, transportation and confinement of white rhinoceroses caused significant stress to the animals, as reflected by HPA activity. We could not establish any predictor of success of habituation in the boma environment. While the rhinoceroses were in confinement management practices contributed to HPA axis responses in the rhinoceroses. This highlights the

importance of continuing physiological assessment of the impact of management practices for all rhinoceroses held in confinement.

CHAPTER 5

Acute Phase Protein Response to ACTH challenge and capture, confinement and translocation of the white rhinoceros

In an attempt to quantify the stress experienced by rhinoceroses during capture, confinement and translocation, we have previously measured FGM (Chapter 4). This procedure is non-invasive, relevant and robust but the time required to obtain the final data could take several days. Data gathered in this way is applicable to monitor the stress of rhinoceroses in confinement, but has no application where rhinoceroses are captured and immediately translocated to other areas. It would therefore be advantageous to be able to relate a stress-induced physiological variable, measured shortly after a relevant sample is obtained at the time of capture, to determine the fate of a translocated animal. The capture and translocation of the white rhinoceros involves physical exertion, handling and transportation to a new environment which all contribute to physical, physiological and psychological stressors. Individually or combined, these stressors may have detrimental effects on the animal (Dickens et al., 2010).

Besides the usual neuro-hormonal response to stress, studies of human (Kang and Fox, 2001), rodent (Hale et al., 2003; Nukina et al., 2001) and domestic animal responses to psychological stress have also highlighted the role of the pro-inflammatory cytokines. These cytokines, secreted from activated macrophages are able to modulate protein synthesis by the hepatocytes, resulting in secretion of a number of proteins in the so-called acute phase response (APR). The goal of the APR is to re-establish homeostasis and to promote healing and is part of the innate immune response. Acute phase proteins (APP) are blood proteins synthesized by hepatocytes during the APR and are triggered by stimuli like trauma, infection, stress, neoplasia and inflammation (Carroll and Forsberg, 2007). The APR may result in changes in more than 200 proteins which can be grouped as positive APP and negative APP. Albumin and transferrin are major negative APP which decrease in concentration in the blood during the APR. Positive APP

concentrations increase in the blood during the APR and can be further classified as major, moderate or minor, depending on the magnitude of their increase (Cray, 2012). Major proteins are those that increase 10- to 1000-fold after the triggering event, increase within the first 48 hours of the triggering event and have a very short half-life. Moderate (5- to 10-fold increase) and minor (only slight increase less than twofold) proteins increase slowly during the APR and have a more prolonged duration dependent on the status of the triggering event and are more often observed during chronic processes. The positive APP have multiple functions, which include modulating the immune system, protein transport and tissue protection from the damages of inflammation (Cray et al., 2009). The serum concentration of the APP returns to basal when the triggering factor is no longer present (Petersen et al., 2004).

Currently, veterinary medicine uses APP concentrations to assist with prognosis of diseases in companion and domesticated animals (Cray et al., 2009). However, several recent reports have shown that APP are relevant as biomarkers of stress and indicators of stress (Cray, 2012; Cray et al., 2013). Two components of the rhinoceros capture process, namely the exercise and transportation, are known to raise the plasma cortisol concentrations in several other species (Saeb et al., 2010; Nagata et al., 1999; Schmidt et al., 2010). Previous research showed that serum amyloid A (SAA) and haptoglobin (Hp) are two APP associated with exercise and transportation in endurance horses (Valle et al., 2015; Cywinska et al., 2010). In cattle, elevating the blood cortisol concentration to levels experienced during stress also activates the APR, with an increase in both circulating tumour necrosis factor (TNF)-α and Hp (Cooke et al., 2012). Exercise also increases pro-inflammatory cytokines in horses (Donovan et al., 2007), leading to possible activation of the APR. Transportation of horses has been shown to increase the plasma concentration of Hp and SAA (Casella et al., 2012). Measurement of APP is thus considered to be a rapid diagnosis to evaluate animal welfare under stressful conditions (Gómez-Laguna et al., 2011). This factor is of considerable importance since rapid point-of-care analyses for some APP are already in

advance stages of development (Dr. Pitts personal communication from Tri-Delta Corporation, Ireland and SOMA Bioscience Ltd., UK).

In this study in white rhinoceroses, we investigated if an increase in plasma cortisol concentration, achieved by means of an intramuscular injection of an ACTH analogue (Synacthen®) caused an increase in the plasma concentrations of the two acute phase proteins SAA and Hp. In addition, the APR of SAA and Hp to capture and confinement of wild white rhinoceroses was also investigated.

5.1 Materials and methods

To establish whether an elevation in the serum concentration of SAA and Hp can be induced by an increase in the plasma cortisol concentration in the white rhinoceros, an ACTH challenge was conducted in boma-confined well-adapted rhinoceroses. To determine whether the stress of capture, handling and transport of the white rhinoceros induces an APR, blood samples were obtained from wild-captured rhinoceroses. The samples were obtained immediately after capture in the wild and then again after the rhinoceroses were handled, loaded and transported to the bomas. A final sample was obtained after a period of boma confinement when the rhinoceroses were reloaded from the boma for translocation or release.

5.1.1 Animal subjects

Two trials were used in this study to evaluate both SAA and Hp as indicators of stress in white rhinoceroses. The four white rhinoceroses captured in the wild (see Chapter 2) were used in a cross-over protocol to investigate APR in response to an ACTH challenge. The plasma cortisol response to this procedure in white rhinoceroses was reported on previously (See Chapter 3), where the administration of the ACTH analogue induced an 8-fold increase in plasma cortisol within 15 – 20 minutes.

Secondly, white rhinoceroses were captured in the wild (see Chapter 2), as part of the SANParks annual translocation program and were transported to the bomas in Skukuza in the Kruger National Park. These animals were used to assess the APR following capture. In this trial, ten rhinoceroses were selected by means of a three-category scoring system to assess adaptation to bomas (see chapter 4, Fig. 4.1). The system is based on appetite, faecal consistency and volume, and behaviour of the rhinoceroses in the boma. The rhinoceroses were scored once a day from the first day of arrival after capture in the wild in the boma to the last day when the rhinoceroses were loaded for translocation. A mean score was calculated for each animal for the duration of the confinement period (Miller et al., 2016). The five poorest performing rhinoceroses (five animals with lowest mean score <7) were compared to the five best performing rhinoceroses (five animals with the highest mean score >17) using the scoring system (Table 5.1).

Table 5.1 Information and mean score of 10 captured and confined white rhinoceroses.

Animal no	Sex	Age (years)	Mean score	
31/09	Female	5-7	19.02	Best performing group
22/09	Male	5-7	18.18	
39/09	Male	5-7	17.82	
43/09	Female	5-7	17.57	
37/09	Female	5-7	17.49	
2/12	Male	5-7	6.76	Poor performing group
10/12	Male	5-7	6.88	
6/12	Male	4-5	6.93	
9/11	Female	4-5	8.18	
10/11	Male	5-7	8.24	

The five poorest performing rhinoceroses were released back into the wild within 10-12 days because of lack of adaptation to the captive conditions. The other rhinoceroses were kept in the bomas for between 120-180 days in preparation for translocation.

5.1.2 Sample collection and ACTH challenge trial

During the ACTH challenge blood samples were collected from the auricular vein once the rhinoceros was recumbent making use of an intravenous catheter (detail description in Chapter 3).

For this study, the APP concentrations at T0 and T60 were compared to establish if there were any significant differences after exposure to elevated plasma cortisol for this length of time. Sixty minutes was also the average time (range 30 to 90 minutes) it took to transport the rhinoceroses from capture sites in the wild to where they were released into a boma (see Chapter 2).

5.1.3 Capture, transport and confinement trial

The capture and sample collection procedures for this part of the study were described in detail in Chapter 2.

5.1.4 Haptoglobin assay

The Hp assay (Tri-Delta Development Limited, County Kildare, Ireland, Cat. No. TP-801) is a colorimetric assay designed to quantitatively measure the concentration of Hp in serum or in plasma in a wide range of animal species. Under normal conditions Hp is either absent from the blood or present at very low concentrations. Hp can however increase significantly in response to acute infection, inflammation or trauma (Tridelta, 2017a).
The principle of the assay is that free Hp exhibits peroxidase activity at a low pH. The Hp in a specimen binds to haemoglobin and at a low pH preserves the

peroxidase activity of the bound haemoglobin. The peroxidase activity of the specimen is then directly proportional to the amount of Hp in the specimen (Tridelta, 2017a).

5.1.5 Serum Amyloid A assay

The SAA assay kit detects SAA in serum or in plasma in a wide range of animal species. It is a solid phase sandwich Enzyme Linked Immunosorbent Assay (ELISA) (Tri-Delta Development Limited, County Kildare, Ireland, Cat. No. TP-802). A monoclonal antibody specific for SAA has been coated into wells with a HRP labelled anti-SAA antibody. Any SAA present in a sample will be captured between the coat of microplate and the labelled antibody. After washing the plate and the addition of the TMB substrate, a blue coloration that is proportional to the amount of SAA present in the sample is generated. The reaction is then stopped with a low pH reagent. For the assay the manufacturer-recommended methodologies were followed (Tridelta, 2017b).

5.1.6 Plasma Cortisol assay

Plasma cortisol concentrations were measured using a commercially available ^{125}I RIA kit (Coat-a-Count®, Siemens Healthcare Diagnostics, Los Angeles, Santa Ana CA 90045, USA) as previously described (see Chapter 3).

5.1.7 Data analysis

ACTH trial: A one-way ANOVA with repeated measures as well as the Tukey's multiple comparison post-test was used to compare plasma concentrations of Hp, SAA and cortisol in the ACTH trial and within group comparisons in the capture, transport and confinement trial.

Captured Rhinoceroses: For the comparison of plasma concentrations of Hp and SAA within and between groups in the capture, transport and confinement trial, a one-way ANOVA as well as the Tukey's multiple comparison post-test was used.

To determine whether there is any relationship between plasma cortisol concentration and SAA secretion, a Pearson correlation analysis was applied to the data.

5.2 Results

5.2.1 Haptoglobin

There was no significant difference in the Hp concentrations between T0 and T60 when ACTH was administered or when saline was administered in the four rhinoceroses subjected to the ACTH challenge (Figure 5.1). Thus, neither 60 minutes of immobilization nor 60 minutes of exposure to an elevated plasma cortisol concentration produced an increase in Hp concentrations in these rhinoceroses. Figure 5.2 shows the plasma Hp concentrations in the captured rhinoceroses assigned to the poor performing and best performing groups. The Hp concentrations in the samples collected from the rhinoceroses at the capture (C1) and in the samples collected from the rhinoceroses at the time they were released

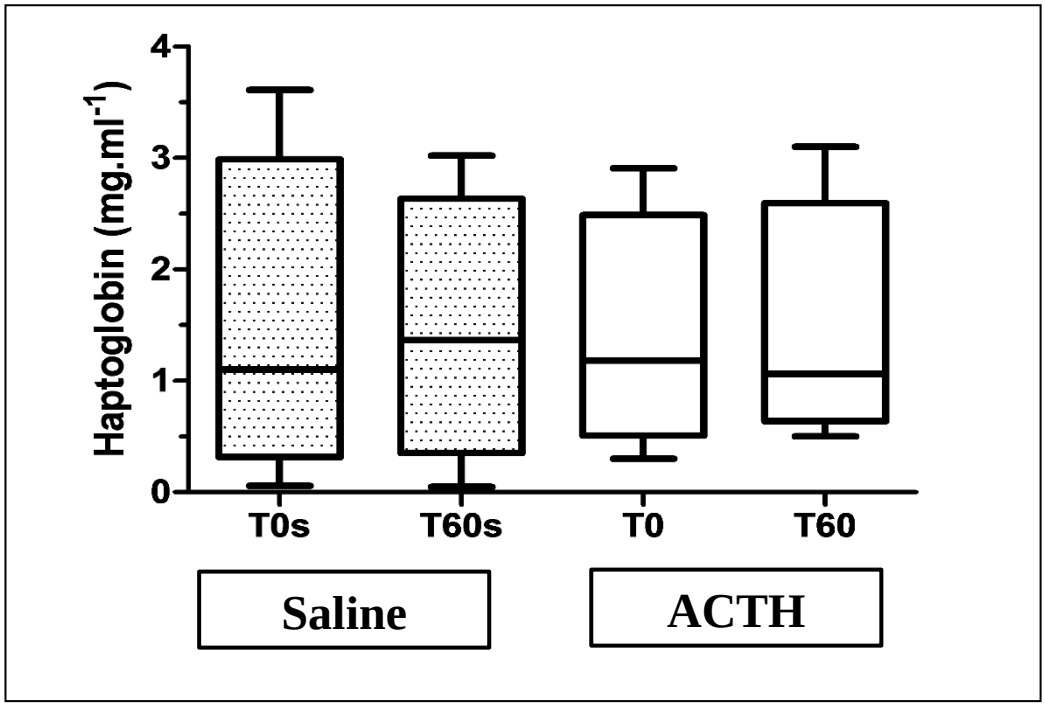


Figure 5.1 Plasma haptoglobin response to saline or ACTH administration.

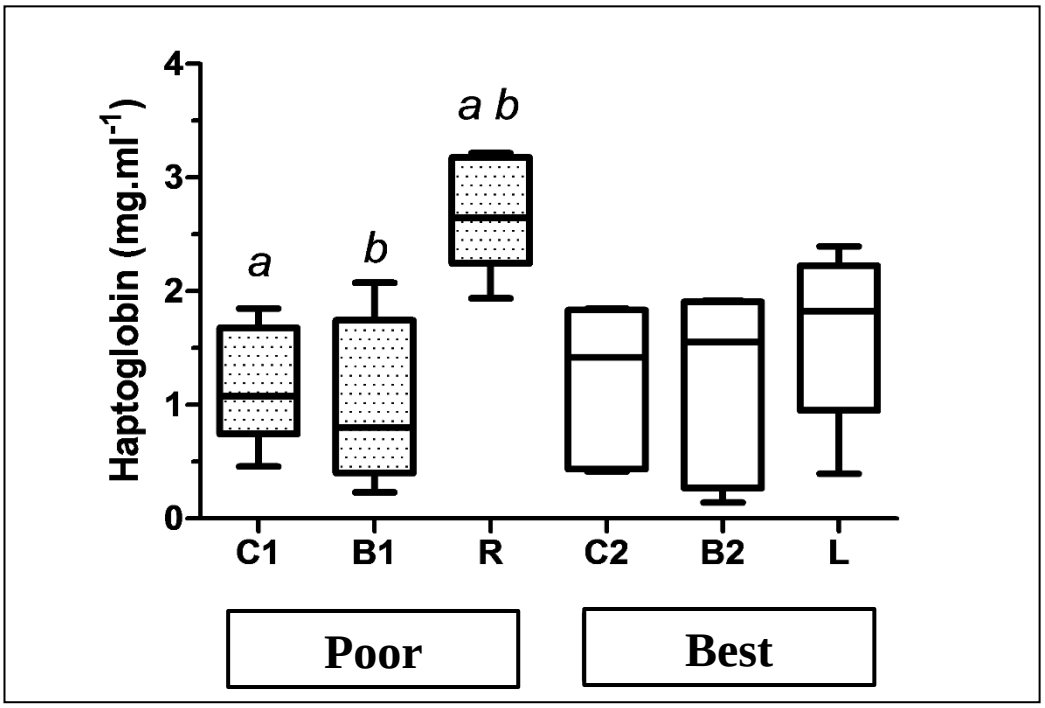


Figure 5.2 Haptoglobin response to capture, transportation and confinement.
Note: Columns with the same letter (a or b) are significantly different from each other ($P<0.01$; $n=5$).

(R) because of poor performance differ significantly ($P < 0.05$). There was also a significant difference in the Hp concentrations of these rhinoceroses at the time when they were offloaded at the boma after capture and transportation of 60 minutes (B1) compared to when they were released (R) ($P < 0.01$). In the rhinoceroses assigned to the best performing group, there are no significant difference in the Hp concentrations in the different samples obtained at similar time points. However, even in the well-adapted animals there was a tendency for the Hp to be increased after confinement ($P=0.059$). There were no significant differences in the plasma Hp concentration between the groups at each time point, including when the rhinoceroses were either released or reloaded for translocation.

These results indicate that neither 60 minutes of immobilization nor 60 minutes of exposure to elevated plasma cortisol concentration, whether from the physical capture and transport or ACTH challenge, have an effect on plasma Hp concentration.

5.2.2 Serum Amyloid A

There were significant differences in the SAA concentrations between T0 and T60 when ACTH was administered and when saline was administered ($P < 0.001$) in the four rhinoceroses subjected to the ACTH challenge (Figure 5.3). In addition, there was a significant difference between the two samples collected at T60s and T60 ($P < 0.001$). This means that 60 minutes of immobilization alone did significantly ($P < 0.001$) increase the SAA concentration. A further significant increase in SAA was seen in the ACTH challenge, where the combined effects of 60 minutes' immobilization plus the elevated cortisol concentration are present.

In the captured rhinoceroses assigned to the poor performing group, there were no significant differences in SAA concentrations at any time point (Figure 5.4). Within the best performing group, the SAA concentrations differ significantly between the samples collected at capture (C2) and those samples collected when

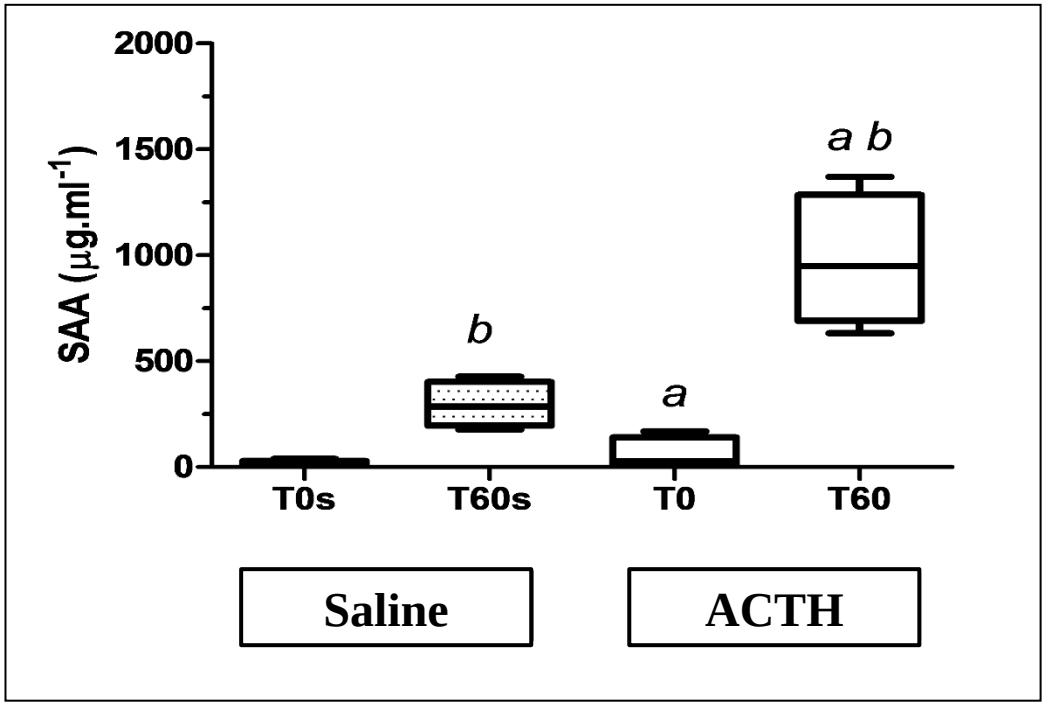


Figure 5.3 SAA response to saline or ACTH administration.
Note: Columns with the same letter (a and b) are significantly different to each other.

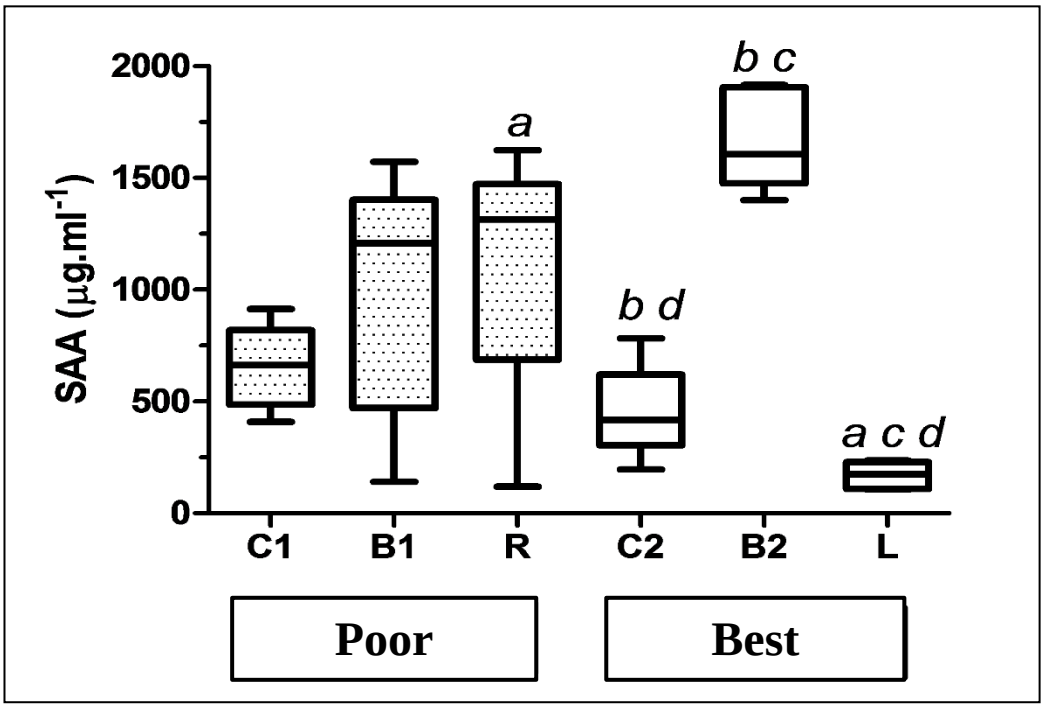


Figure 5.4 SAA response to capture, transportation and confinement.
Note: Columns with the same letter (a, b, c or d) are significantly different from each other.

the rhinoceroses were offloaded at the boma after the capture and transportation of 60 minutes (B2; $P < 0.001$). After the period of successful confinement (L), where the rhinoceroses adapted to confinement, the SAA concentrations were lower than those at capture (C2; $P<0.05$) and at release into the boma (B2; $P<0.001$). When comparing between the poor performing group and the best performing group, the SAA concentrations were significantly ($P< 0.001$) higher (R) in the poor performing group when they were released than in the best performing group when they were loaded after successful confinement (L) (Figure 5.4). It is also important to note that the average SAA concentration from the rhinoceroses darted in the bomas for the ACTH challenge was initially significantly lower than that measured in the ten wild-captured rhinoceroses at capture ($P<0.0001$)

5.2.3 Plasma Cortisol

The plasma cortisol concentrations at capture, offloading at the boma and when the rhinoceroses were released from the boma differed between the poor

performing group and the best performing group (Figure 5.5). Within the poor performing group, the cortisol concentrations of the samples collected at capture (C1) and that of the samples collected after capture and translocation of 60 minutes (B1) differ significantly ($P < 0.001$). The cortisol concentrations also differed significantly between samples collected after capture and transportation of 60 minutes (B1) and those obtained when the rhinoceroses were released (R) because of poor performance ($P < 0.01$). Within the best performing group, the cortisol concentrations of the samples collected at capture (C2) and that of the samples collected after capture and 60 minutes of transportation (B2) differ significantly ($P < 0.05$). There was also a difference in the samples collected at capture and 60 minutes of transportation (B2) and that of when the rhinoceroses were loaded for translocation after a period of confinement (L) ($P < 0.05$).

From these results, it can be seen that there was a significant increase in plasma cortisol concentrations in both groups after capture and subsequent 60 minutes of

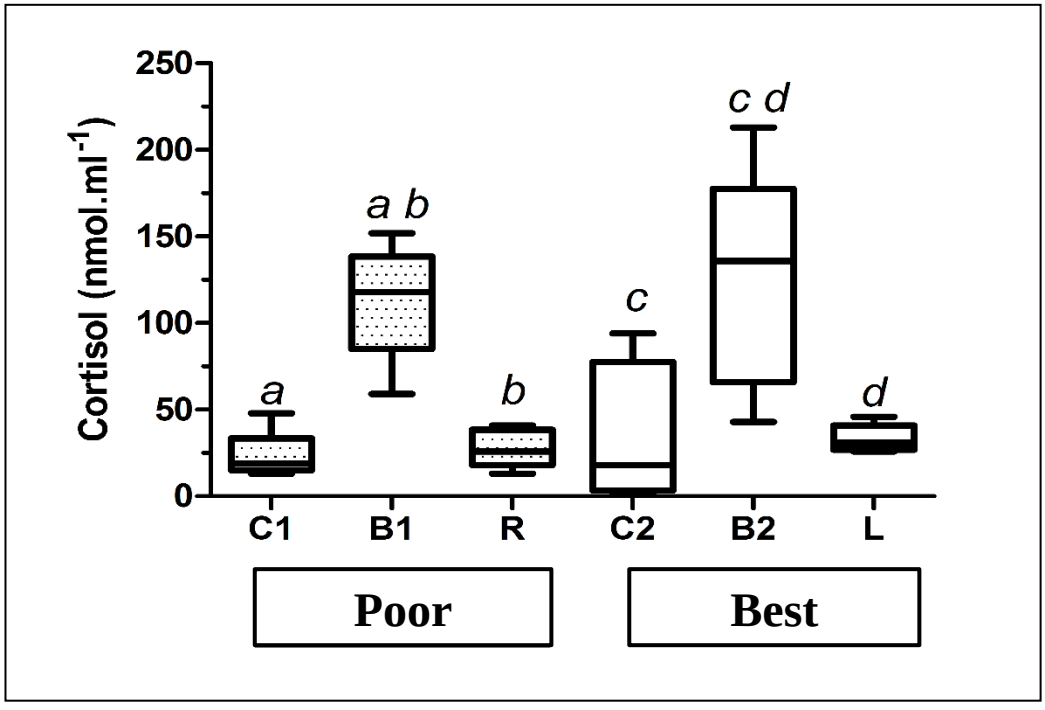


Figure 5.5 Plasma cortisol response to capture, subsequent transportation and confinement.

Note: Columns with the same letter (a, b, c or d) are significantly different from each other.

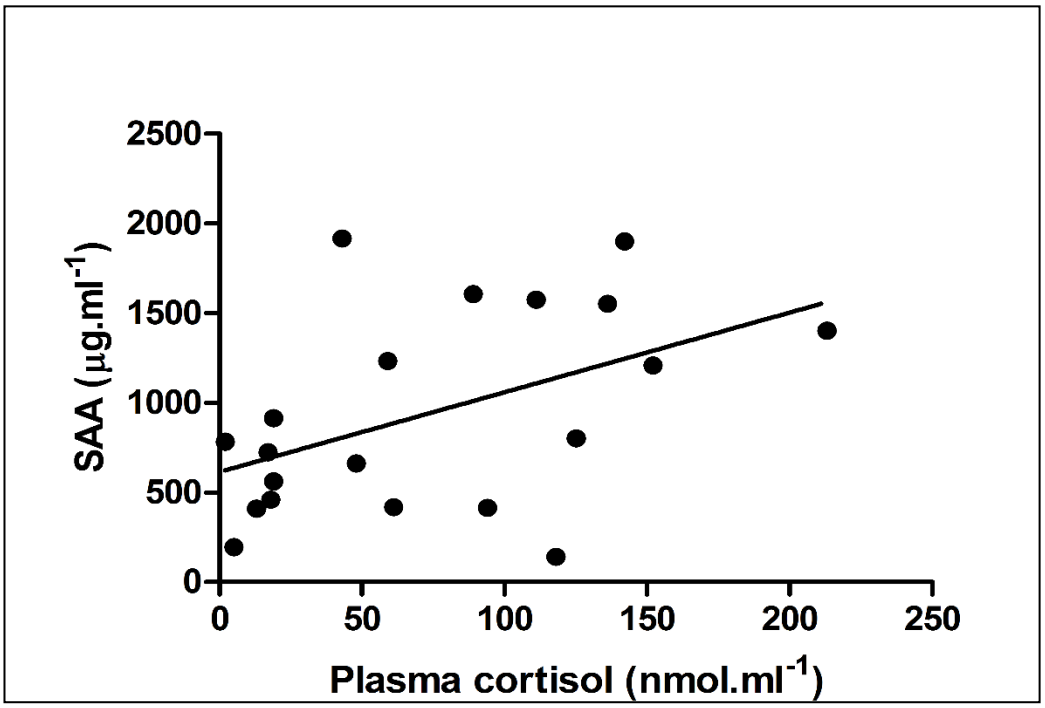


Figure 5.6 Correlation between plasma cortisol and plasma SAA; Pearson r = 0.4740; P=0.0347; n=20.

transportation, but that there are no significant differences in plasma cortisol concentrations between the two groups at each sample time.

Figure 5.6 shows the relationship between plasma cortisol and SAA concentration for the ACTH trial and the ten wild-captured rhinoceroses at all time points. There was a significant correlation between plasma cortisol and the SAA concentration in the combined data set (Pearson r = 0.4740; P=0.0347; n=20). There was no significant correlation between plasma cortisol and the Hp concentration in the combined data set (Pearson r = -0.2988; P=0.2007; n=20).

5.3 Discussion

From the results above, it would appear that the changes in SAA concentration are more sensitive to the effects of immobilization and to the exposure of elevated plasma cortisol caused by capture, immobilization and transportation than Hp in white rhinoceroses.

Immobilization for 60 minutes increased SAA, and the further addition of an ACTH-induced increase in plasma cortisol caused a 3-fold increase in SAA. This suggests that circulating SAA was acutely influenced by the prevailing cortisol concentration. In contrast, plasma Hp concentrations showed no such effects. Thus, it would appear that activation of the HPA axis by stress-related incidents was also associated with an increase in SAA. This association between plasma cortisol concentration and SAA concentrations was further confirmed by the significant positive Pearson's correlation between these parameters in the capture, transport and confinement trial (Figure 5.6).

However, other studies have noted that there appears to be a time dependent effect on the rate of increase in these two acute phase proteins. For example, acute corticotropin-releasing hormone (CRH) administration also elicits a pro-inflammatory response in conscious cattle (Hulbert et al., 2013) but the Hp response has been shown to only peak 72 hours after CRH infusion (Cooke et al., 2012). The differences in the kinetics of these two proteins means that it is not accurate to compare changes in concentration at the same time point after imposition of the stressor.

How does this HPA-associated increase in SAA then relate to the stress experienced by white rhinoceroses captured in the wild and then transported to the bomas for a period of confinement? In the group of rhinoceroses that showed good adaptation to the confinement, the stress of capture and transport was associated with a significant increase in SAA, while Hp showed no response. However, in the rhinoceroses that had to be released due to lack of adaptation, varied individual responses resulted in a lack of significant increase in SAA. Despite the lack of a significant increase in SAA over the initial period of capture and transportation, the rhinoceroses which were released due to poor adaptation showed signs of an APR that did not resolve over time, but rather increased during confinement.

Earlier work showed that an increase in SAA has been associated with transportation of animals (Arthington et al., 2003; Fazio et al., 2015; Casella et al., 2012; Lomborg et al., 2008) and exercise (Valle et al., 2015). Transportation following physical restraint also has an enhanced effect on the stress response in animals (López-Olvera et al., 2006) and stressors associated with transportation and new accommodation can cause an increase in APP (Salamano et al., 2008). This idea is further supported by the fact that the plasma Hp concentration in the current study continued to rise during confinement. Such increases in Hp have been shown when pigs were deprived of food for 24 hours (Ott et al., 2014). Hp would therefore appear to be a more sensitive indicator of metabolic stress rather than to a psychological stress. A similar finding was made in calves where a physical stressor increased the SAA concentration but had no effect on the plasma Hp concentration (Alsemgeest et al., 1995).

A further feature of the SAA response can be noted in the marked differences observed between the ten wild-captured rhinoceroses and the four boma-habituated rhinoceroses used in the ACTH trial. There were no differences in the Hp concentration between these groups, but the results either indicate that the SAA concentration is higher in free-living rhinoceroses than in boma-adapted rhinoceroses, or that the SAA response is extremely sensitive to the physical and/or psychological stressors associated with the initial capture phase of the operation. A study on manatees has also shown that exposure to trauma or colder water resulted in significantly higher SAA concentrations than other animals at capture due to these additional physical stressors (Wong et al., 2012). This theory is further supported by the fact that when the best performing rhinoceroses were darted in the boma for translocation, their SAA concentrations were significantly lower than those concentrations recorded at capture. The APR to transport is known to decline as the animals habituate to their new environment (Berry et al., 2004).

Is the SAA response to capture, transport and confinement associated with differences in HPA reactivity in the groups that show poor and better habituation?

It has long been recognized that glucocorticoids are partly responsible for the secretion of hepatic APP like SAA (Baumann et al., 1989). In the current study both groups show similar cortisol responses to the capture and transport, it can therefore not be confirmed that cortisol was the only factor that contributed to the observed SAA response. This finding may be due to the small sample numbers. However, when the correlation between plasma cortisol and SAA concentrations in all the blood samples was examined (i.e. capture and boma data for both groups), it shows that plasma cortisol was positively associated with SAA secretion. This significant agreement between plasma cortisol concentration and SAA concentration has been confirmed in pigs subjected to the stressors of social isolation and short road transport (Soler et al., 2013). Even though the sample size in this rhinoceros study was small, the significant differences in SAA observed when the rhinoceroses are unloaded at the boma suggest that this biomarker may be of use in identifying rhinoceroses that could be at risk of failing to habituate to confinement. This finding warrants further investigation.

Another area of investigation should examine the effects of longer periods of confinement on the APR in the white rhinoceros. Even in the group of best performing rhinoceroses there was a tendency for Hp to increase compared to the levels obtained at capture ($P= 0.059$). In zoo-managed black rhinoceroses concentrations of SAA are elevated compared to free-living rhinoceroses (Schook et al., 2015). This previous zoo animal study also concluded that the captive environment could be contributing to increased inflammation and metabolic derangements in captive rhinoceroses, which may later lead to increased risk of developing insulin resistance and diabetes.

It is uncertain what exact role SAA plays in affording protection from the effects of capture, transportation and confinement in these rhinoceroses. However, a review of possible amyloid function concluded that SAA must have a very important physiological role, since SAA genes have existed for at least 500 million years and that SAA's amino acid sequence has remained largely conserved over this period of time (Kisilevsky and Manley, 2012). A similar

conclusion was made by Ye and Sun (2015) who proposed that SAA has a homeostatic role during the course of inflammation. One specific mechanism of its function is that it binds with HDL, increasing the anti-oxidant potential of these molecules (Sato et al., 2016). It is thus hypothesized that increased levels of SAA contribute to boma habituation success through the role it plays to maintain homeostasis during inflammatory responses that may occur during the confinement period.

It is furthermore proposed that; in the white rhinoceroses; where the SAA concentrations significantly increase as a result of capture and immobilization and the subsequent transportation in the wild, there is an increased chance of successful habituation to confinement. This initial finding needs to be confirmed by firstly expanding the number of rhinoceroses in the study group, and secondly by investigating the effectiveness of on-site testing kits in determining SAA concentrations in the rhinoceros at capture.

CHAPTER 6

Conclusion

Animal numbers over the entire continent are diminishing because of anthropogenic impacts. In light of this, conservation efforts to prevent the loss in biodiversity need to be intensified in order to facilitate the recovery of the endangered species. Almost three rhinoceroses are being poached every day in South Africa alone. Poaching of rhinoceroses in South Africa increased from 13 in the year 2007 to 1215 in 2014 and 1175 in 2015 (Savetherhino, 2017). At this rate both the black and white rhinoceros populations will be destroyed within a few years. By translocating and re-introducing rhinoceroses into other areas we may save the population from extinction. Operations like these are, however accompanied by stress on the animals which may strongly affect the successful outcome of the operation. In our study, we validated a technique to measure adrenocortical responses in white rhinoceroses. We also assessed the stress associated with capture, handling, confinement and transportation of white rhinoceroses, with the ultimate goal being to be able to determine an animal's fate at the time of capture and translocation.

Other research initiatives which ran concurrent to and were associated with this study included; biochemical values of free ranging white rhinoceroses (Mathebula et al., 2012), the use of butorphanol in the immobilization of free-ranging white rhinoceroses (Miller et al., 2013) and the development of a scoring system to improve decision-making in the adaptation of white rhinoceroses (Miller et al., 2016).

In this study, we concurred that the stimulation of the HPA axis through a single intra-muscular injection of a synthetic analogue of ACTH, Synacthen®, produced a significant increase in the plasma cortisol concentration. This subsequently induced a significant increase in the FGM concentration in the white rhinoceroses. The dose of Synacthen® also resembles the response to acute stress caused by the

capture and immobilization processes (Kock et al., 1990). Data from wild-captured white rhinoceroses confined to the boma confirmed that physiological/psychological stressors associated with capture and translocation are sufficient to produce measurable increases in the FGM concentration in rhinoceroses.

We found that white rhinoceroses have variable responses to capture. This suggests that there are individual differences between animals in response to handling and transportation after capture. White rhinoceroses show low concentrations of FGM at the time of capture, but this increases within four days of confinement after capture. This is an indication of strong stimulation of the HPA axis. This initial stimulation is followed by an average decline in FGM concentrations between 14 to 17 days. A further point of interest noted was that the FGM concentration does not return to pre-capture levels when the rhinoceroses are confined for extended periods. There is thus evidence of behavioural habituation but HPA axis activity shows that there is in fact no physiological habituation.

Our study also showed that under conditions of increased blood cortisol concentration it can be expected that gastrointestinal transit time is extended and may be associated with reduced feed intake. In addition, the extended gastrointestinal transit time should be considered when evaluating stressed rhinoceroses to ensure that the measurement of FGM corresponds with the timing of the stressor being evaluated.

Results from this study also showed that it is important to minimize the stress responses through good planning and management. The design of the holding facilities is very important for success and goes hand in hand with intensive management of rhinoceroses in confinement.

Although FGM measurement is non-invasive, the time required to complete the extraction and assay takes several days and it cannot be used to predict a

rhinoceros's fate at the time of capture. For this purpose, a more rapid field assessment is required. Our study on acute phase proteins (APP) and the acute phase response (APR) showed that it may in future be the tool needed to predict the fate of an animal at the time of capture. APP concentrations are currently used to assist with prognosis of diseases in companion and domesticated animals (Cray et al., 2009). Several reports have also shown that APP are relevant as biomarkers and indicators of stress (Cray, 2012; Cray et al., 2013) and that serum amyloid A (SAA) and haptoglobin (Hp) are two APP associated with exercise and transportation in endurance horses (Valle et al., 2015; Cywinska et al., 2010).

It would appear, from our study, that the changes in SAA concentration is more sensitive to the effects of immobilization and to the exposure of elevated plasma cortisol caused by capture, immobilization and transportation. Hp seems to be a more sensitive indicator of metabolic stress rather than psychological stress. It was found that immobilization of 60 minutes increased SAA. When this immobilization is also accompanied by an ACTH-induced increase in plasma cortisol, there is a further 3-fold increase in SAA. This suggests that circulating SAA is acutely influenced by the prevailing cortisol concentration. However, Hp shows no such effects.

How does this HPA-associated increase in SAA then relate to the stress experienced by white rhinoceroses captured in the wild and then transported to the bomas for a period of confinement? Our study showed that in a group of rhinoceroses that showed good adaptation to the confinement, the stress of capture and transport was associated with a strong SAA response. Another group of rhinoceroses that had to be released due to lack of adaptation showed varied individual responses that resulted in a lack of significant increase in SAA, with signs of an APR that did not resolve over time.

Another question asked was; is the SAA response to capture, transport and confinement associated with differences in HPA reactivity in the groups that show poor and better habituation? Our study showed that both groups presented similar

cortisol responses to the capture and transport, it can therefore not be confirmed that cortisol is the only factor which contributes to the observed SAA response. When the correlation between plasma cortisol and SAA concentrations in all the blood samples were examined, it however, showed that plasma cortisol is positively associated with SAA secretion. Another area of investigation in future should examine the effects of longer periods of confinement on the APR in the white rhinoceros.

It would appear from our work that successful adaptation to confinement in white rhinoceroses was associated with a significant increase in SAA concentrations in response to capture and immobilization in the wild and the subsequent transportation to a holding facility. This initial finding needs to be confirmed by expanding the number of rhinoceroses in the study group, and by investigating the effectiveness of on-site testing kits in determining SAA concentrations in the rhinoceros. The significant differences in SAA concentrations observed in rhinoceroses in this study after capture in the wild, suggest that this biomarker may in future be used to identify rhinoceroses at risk of failing to habituate to confinement. This however warrants further investigation.

This study on white rhinoceroses also delivered some unique achievements. In validating a non-invasive assessment of stress in white rhinoceroses a comprehensive ACTH challenge was done for the first time on white rhinoceroses. It was also the first time an assessment was done on the capture variables that may influence the successful outcome of white rhinoceros confinement. The study furthermore also used FGM to identify management issues in the boma placement of rhinoceroses related to dominance structures and social behaviour that was not done before. It was also the first time that the measurement of SAA and Hp was used after an ACTH challenge, as well as at capture and after transportation of white rhinoceroses. In doing so a possible biomarker to determine the fate of a white rhinoceros at the time of capture was identified. Lastly, it paved the way for future research in this field to improve the

management of rhinoceroses in confinement and to ensure successful translocation to save this species from extinction.

REFERENCES

Alsemgeest, S., Lambooy, I., Wierenga, H., Dieleman, S., Meerkerk, B., Van Ederen, A. & Niewold, T. A. (1995). Influence of physical stress on the plasma concentration of serum amyloid-a (SAA) and haptoglobin (HP) in calves. *Veterinary Quarterly,* 17**,** 9-12.

Arias, N., Requena, M. & Palme, R. (2013). Measuring faecal glucocorticoid metabolites as a non-invasive tool for monitoring adrenocortical activity in South American camelids. *Animal Welfare,* 22**,** 25-31.

Armario, A. (2006). The Hypothalamic-Pituitary-Adrenal Axis: What can it Tell us About Stressors? *CNS & Neurological Disorders - Drug Targets (Formerly Current Drug Targets,* 5**,** 485-501.

Armstrong, D. P. & Seddon, P. J. (2008). Directions in reintroduction biology. *Trends in Ecology & Evolution,* 23**,** 20-25.

Arthington, J. D., Eicher, S. D., Kunkle, W. E. & Martin, F. G. (2003). Effect of transportation and commingling on the acute-phase protein response, growth, and feed intake of newly weaned beef calves. *Journal of Animal Science,* 81**,** 1120-1125.

Baumann, H., Prowse, K., Marinković, S., Won, K. A. & Jahreis, G. (1989). Stimulation of hepatic acute phase response by cytokines and glucocorticoidsa. *Annals of the New York Academy of Sciences,* 557**,** 280-296.

Bayazit, V. (2009). Evaluation of cortisol and stress in captive animals. *Australian Journal of Basic and Applied Sciences,* 3**,** 1022-1031.

Belda, X., Fuentes, S., Nadal, R. & Armario, A. (2008). A single exposure to immobilization causes long-lasting pituitary-adrenal and behavioral sensitization to mild stressors. *Hormones and Behavior,* 54**,** 654-661.

Belda, X., Márquez, C. & Armario, A. (2004). Long-term effects of a single exposure to stress in adult rats on behavior and hypothalamic–pituitary–adrenal

responsiveness: comparison of two outbred rat strains. *Behavioural Brain Research,* 154**,** 399-408.

Berga, S. L. (2008). Stress and Reprodution: A Tale of False Dichotomy? *Endocrinology,* 149**,** 867-868.

Berry, B. A., Confer, A. W., Krehbiel, C. R., Gill, D. R., Smith, R. A. & Montelongo, M. (2004). Effects of dietary energy and starch concentrations for newly received feedlot calves: II. Acute-phase protein response12. *Journal of Animal Science,* 82**,** 845-850.

Brambilla, F. (2001). Social stress in anorexia nervosa: a review of immuno-endocrine relationships. *Physiology and Behaviour* 73**,** 365-369.

Breazile, J. (1987). Physiological basis and consequences of distress in animals. *Journal of the American Veterinary Medical Association,* 191**,** 1212-1215.

Brown, J. L., Bellem, A. C., Fouraker, M., Wildt, D. E. & Roth, T. L. (2001). Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. *Zoo Biology,* 20**,** 463-486.

Budzyńska, M. (2014). Stress Reactivity and Coping in Horse Adaptation to Environment. *Journal of Equine Veterinary Science,* 34**,** 935-941.

Capiro, J. M., Stoops, M. A., Freeman, E. W., Clawson, D. & Schook, M. W. (2014). Effects of management strategies on glucocorticoids and behavior in Indian rhinoceros (Rhinoceros unicornis): Translocation and operant conditioning. *Zoo Biology,* 33**,** 131-143.

Carlstead, K. & Brown, J. L. (2005). Relationships between patterns of Fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (Diceros bicornis) and white (Ceratotherium simum) rhinoceros. *Zoo Biology,* 24**,** 215-232.

Carroll, J. A. & Forsberg, N. E. (2007). Influence of Stress and Nutrition on Cattle Immunity. *Veterinary Clinics of North America: Food Animal Practice,* 23**,** 105-149.

Casella, S., Fazio, F., Giannetto, C., Giudice, E. & Piccione, G. (2012). Influence of transportation on serum concentrations of acute phase proteins in horse. *Research in Veterinary Science,* 93**,** 914-917.

Chinnadurai, S. K., Millspaugh, J. J., Matthews, W. S., Canter, K., Slotow, R., Washburn, B. E. & Woods, R. J. (2009). Validation of Fecal Glucocorticoid Metabolite Assays for South African Herbivores. *Journal of Wildlife Management,* 73**,** 1014-1020.

Chrousos, G. P. & Gold, P. W. (1992). The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *Jama,* 267**,** 1244-52.

Cockrem, J. F. (2013). Individual variation in glucocorticoid stress responses in animals. *General and Comparative Endocrinology,* 181**,** 45-58.

Cooke, R. F., Carroll, J. A., Dailey, J., Cappellozza, B. I. & Bohnert, D. W. (2012). Bovine acute-phase response after different doses of corticotropin-releasing hormone challenge. *Journal of Animal Science,* 90**,** 2337-2344.

Cooke, S. J., Blumstein, D. T., Buchholz, R., Caro, T., Fernández-Juricic, E., Franklin, C. E., Metcalfe, J., O'connor, C. M., St Clair, C. C., Sutherland, W. J. & Wikelski, M. (2014). Physiology, behavior, and conservation. *Physiological and biochemical zoology : PBZ,* 87**,** 1-14.

Cray, C. (2012). Acute phase proteins in animals. *Progress in molecular biology and translational science,* 105**,** 113-150.

Cray, C., Hammond, E. & Haefele, H. (2013). Acute phase protein and protein electrophoresis values for captive Grant's Zebra (*Equus burchelli). Journal of Zoo and Wildlife Medicine,* 44**,** 1107-1110.

Cray, C., Zaias, J. & Altman, N. H. (2009). Acute phase response in animals: a review. *Comparative medicine,* 59**,** 517.

Creel, S., Fox, J., Hardy, A., Sands, J., Garrott, B. & Peterson, R. (2002). Snowmobile activity and glucocorticoid stress response in wolves and elk. *Conserv Biol,* 16**,** 809-814.

Cyr, N. E. & Romero, L. M. (2009). Identifying hormonal habituation in field studies of stress. *Gen Comp Endocrinol,* 161**,** 295-303.

Cywinska, A., Gorecka, R., Szarska, E., Witkowski, L., Dziekan, P. & Schollenberger, A. (2010). Serum amyloid A level as a potential indicator of the status of endurance horses. *Equine Veterinary Journal,* 42**,** 23-27.

Dallman, M. F., Akana, S. F., Levin, N., Walker, C. D., Bradbury, M. J., Suemaru, S. & Scribner, K. S. (1994). Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis. *Ann N Y Acad Sci,* 746**,** 22-31; discussion 31-2, 64-7.

Dallman, M. F., Akana, S. F., Scribner, K. A., Bradbury, M. J., Walker, C. D., Strack, A. M. & Cascio, C. S. (1992). Stress, Feedback and Facilitation in the Hypothalamo-Pituitary-Adrenal Axis. *J Neuroendocrinol,* 4**,** 517-526.

De Kloet, E. R. (2003). Hormones, brain and stress. *Endocrine regulations,* 37**,** 51-68.

De Kloet, E. R., Joels, M. & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nat Rev Neurosci,* 6**,** 463-475.

Dehnhard, M., Clauss, M., Lechner-Doll, M., Meyer, H. H. & Palme, R. (2001). Noninvasive monitoring of adrenocortical activity in roe deer (Capreolus capreolus) by measurement of fecal cortisol metabolites. *Gen Comp Endocrinol,* 123**,** 111-20.

Dhabhar, F. S. & Mcewen, B. S. (1997). Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunityin Vivo:A Potential Role for Leukocyte Trafficking. *Brain, Behavior, and Immunity,* 11**,** 286-306.

Di Minin, E., Laitila, J., Montesino-Pouzols, F., Leader-Williams, N., Slotow, R., Goodman, P. S., Conway, A. J. & Moilanen, A. (2015). Identification of policies for a sustainable legal trade in rhinoceros horn based on population projection and socioeconomic models. *Conservation Biology,* 29**,** 545-555.

Dickens, M. J., Delehanty, D. J. & Michael Romero, L. (2010). Stress: An inevitable component of animal translocation. *Biological Conservation,* 143**,** 1329-1341.

Dickens, M. J. & Romero, L. M. (2013). A consensus endocrine profile for chronically stressed wild animals does not exist. *General and Comparative Endocrinology,* 191**,** 177-189.

Dobrakovová, M., Kvetňanský, R., Opršalová, Z. & Jeẑová, D. (1993). Specificity of the effect of repeated handling on sympathetic-adrenomedullary and pituitary-adrenocortical activity in rats. *Psychoneuroendocrinology,* 18**,** 163-174.

Donovan, D. C., Jackson, C. A., Colahan, P. T., Norton, N. & Hurley, D. J. (2007). Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin F2α in horses. *Veterinary Immunology and Immunopathology,* 118**,** 263-269.

Dorsey, C., Dennis, P., Guagnano, G., Wood, T. & Brown, J. L. (2010). Decreased Baseline Fecal Glucocorticoid Concentrations Associated with Skin and Oral Lesions in Black Rhinoceros (Diceros bicornis). *Journal of Zoo and Wildlife Medicine,* 41**,** 616-625.

Esteruelas, N. F., Huber, N., Evans, A. L., Zedrosser, A., Cattet, M., Palomares, F., Angel, M., Swenson, J. E. & Arnemo, J. M. (2015). Leukocyte coping capacity as tool to assess capture-and handling stress in Scandinavian brown bears (Ursus Arctos) *Journal of wildlife diseases,* In-Press.

Fazio, F., Ferrantelli, V., Cicero, A., Casella, S. & Piccione, G. (2015). Utility of acute phase proteins as biomarkers of transport stress in ewes and beef cattle. *Italian Journal of Food Safety,* 4.

Ferreira, S. M., Botha, J. M. & Emmett, M. C. (2012). Anthropogenic Influences on Conservation Values of White Rhinoceros. *PLoS One,* 7**,** e45989.

Ferreira, S. M., Greaver, C., Knight, G. A., Knight, M. H., Smit, I. P. J. & Pienaar, D. (2015). Disruption of Rhino Demography by Poachers May Lead to Population Declines in Kruger National Park, South Africa. *PLoS ONE,* 10**,** e0127783.

Foley, C., Papageorge, S. & Wasser, S. (2001). Non-invasive stress and reproductive measures of social and ecological pressures in free-ranging African elephants (Loxodonta Africana). *Conserv Biol,* 15**,** 1134-1142.

Franceschini, M. D., Rubenstein, D. I., Low, B. & Romero, L. M. (2008). Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. *Animal Conservation,* 11**,** 263-269.

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., Rodríguez-Gómez, I. M., Barranco, I. & Carrasco, L. (2011). Acute Phase Proteins as Biomarkers in Animal Health and Welfare. *In:* VEAS, F. (ed.) *Acute Phase Proteiens as Early Non-Spesific Biomarkers of Human and Veterinary Diseases.* InTech.

Goymann, W., Mostl, E., Van't Hof, T., East, M. L. & Hofer, H. (1999). Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, Crocuta crocuta. *Gen Comp Endocrinol,* 114**,** 340-8.

Graham, L. & Brown, J. (1997). Non-invasive assessment of gonadal and adrenocortical function in felid species via faecal steroid analyses. . *First International Symposium on Physiology and Ethology of Wild and Zoo animals,,* Suppplement II**,** 78-82.

Grissom, N. & Bhatnagar, S. (2009). Habituation to repeated stress: Get used to it. *Neurobiology of Learning and Memory,* 92**,** 215-224.

Hale, K. D., Weigent, D. A., Gauthier, D. K., Hiramoto, R. N. & Ghanta, V. K. (2003). Cytokine and hormone profiles in mice subjected to handling combined with rectal temperature measurement stress and handling only stress. *Life Sciences,* 72**,** 1495-1508.

Harbuz, M. S. & Lightman, S. L. (1992). Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. *J Endocrinol,* 134**,** 327-39.

Haussmann, M. F., Vleck, C. M. & Farrar, E. S. (2007). A laboratory exercise to illustrate increased salivary cortisol in response to three stressful conditions using competitive ELISA. *Adv Physiol Educ,* 31**,** 110-5.

Hawksworth, D. L. & Bull, A. T. (2007). *Biodiversity and conservation in Europe*, Springer.

Hill, S. P. & Broom, D. M. (2009). Measuring zoo animal welfare: theory and practice. *Zoo Biology,* 28**,** 531-544.

Holst, D. V. (1998). The concept of stress and its relevance for animal behavior. *Advances in the study of behavior (USA).*

Huber, S., Palme, R. & Arnold, W. (2003). Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (Cervus elaphus). *Gen Comp Endocrinol,* 130**,** 48-54.

Hulbert, L. E., Carroll, J. A., Ballou, M. A., Burdick, N. C., Dailey, J. W., Caldwell, L. C., Loyd, A. N., Vann, R. C., Welsh, T. H. & Randel, R. D. (2013). Sexually dimorphic stress and pro-inflammatory cytokine responses to an intravenous corticotropin-releasing hormone challenge of Brahman cattle following transportation. *Innate immunity,* 19**,** 378-387.

Hunt, K. E., Trites, A. W. & Wasser, S. K. (2004). Validation of a fecal glucocorticoid assay for Steller sea lions (Eumetopias jubatus). *Physiol Behav,* 80**,** 595-601.

Hunt, K. E. & Wasser, S. K. (2003). Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. *Physiol Biochem Zool,* 76**,** 918-28.

Hutchins, M. & Kreger, M. D. (2006). Rhinoceros behaviour: implications for captive management and conservation. *International Zoo Yearbook,* 40**,** 150-173.

Jacobson, L. (2005). Hypothalamic–pituitary–adrenocortical axis regulation. *Endocrinology and metabolism clinics of North America,* 34**,** 271-292.

Kang, D.-H. & Fox, C. (2001). Th1 and Th2 cytokine responses to academic stress. *Research in Nursing & Health,* 24**,** 245-257.

Keay, J. M., Singh, J., Gaunt, M. C. & Kaur, T. (2006). Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. *J Zoo Wildl Med,* 37**,** 234-44.

Kisilevsky, R. & Manley, P. N. (2012). Acute-phase serum amyloid A: perspectives on its physiological and pathological roles. *Amyloid,* 19**,** 5-14.

Kock, M. D., Toit, R. D., Kock, N., Morton, D., Foggin, C. & Paul, B. (1990). Effects of Capture and Translocation on Biological Parameters in Free-Ranging Black Rhinoceroses (Diceros bicornis) in Zimbabwe. *Journal of Zoo and Wildlife Medicine,* 21**,** 414-424.

Kock, R. A., Mihok, S. R., Wambua, J., Mwanzia, J. & Saigawa, K. (1999). Effects of translocation on hematologic parameters of free-ranging black rhinoceros (Diceros bicornis michaeli) in Kenya. *J Zoo Wildl Med,* 30**,** 389-96.

Koolhaas, J. M., Bartolomucci, A., Buwalda, B., De Boer, S. F., Flügge, G., Korte, S. M., Meerlo, P., Murison, R., Olivier, B., Palanza, P., Richter-Levin, G., Sgoifo, A., Steimer, T., Stiedl, O., Van Dijk, G., Wöhr, M. & Fuchs, E. (2011). Stress revisited: A critical evaluation of the stress concept. *Neuroscience & Biobehavioral Reviews,* 35**,** 1291-1301.

Korte, S. M., Koolhaas, J. M., Wingfield, J. C. & Mcewen, B. S. (2005). The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neuroscience & Biobehavioral Reviews,* 29**,** 3-38.

Korte, S. M., Olivier, B. & Koolhaas, J. M. (2007). A new animal welfare concept based on allostasis. *Physiol Behav,* 92**,** 422-8.

Kruger, M., Pitts, N., Virgo, J., Betts, E., Delk, K. & Fayrer-Hosken, R. (2011). Development of field assay for evaluation of white rhinoceros neutrophil function as a stress marker. *Reproduction Fertility and Development,* 23**,** 181-182.

Levine, S. (2005). Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology,* 30**,** 939-946.

Linklater, W. L., Macdonald, E. A., Flamand, J. R. B. & Czekala, N. M. (2010). Declining and low fecal corticoids are associated with distress, not acclimation to stress, during the translocation of African rhinoceros. *Animal Conservation,* 13**,** 104-111.

Lomborg, S. R., Nielsen, L. R., Heegaard, P. M. & Jacobsen, S. (2008). Acute phase proteins in cattle after exposure to complex stress. *Veterinary research communications,* 32**,** 575-582.

López-Olvera, J. R., Marco, I., Montané, J. & Lavín, S. (2006). Transport stress in Southern chamois (Rupicapra pyrenaica) and its modulation by acepromazine. *The Veterinary Journal,* 172**,** 347-355.

Lynch, J. W., Ziegler, T. E. & Strier, K. B. (2002). Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, Cebus apella nigritus. *Horm Behav,* 41**,** 275-87.

Mathebula, N., Miller, M., Buss, P., Joubert, J., Martin, L., Kruger, M., Hofmeyr, M. & Olea-Popelka, F. (2012). Biochemical values in free-ranging white rhinoceros (Ceratotherium simum) in Kruger National Park, South Africa. *Journal of Zoo and Wildlife Medicine,* 43**,** 530-538.

Mcewan, B. & Stellar, E. (1993). Stress and the individual: Mechanisms leading to disease. *Arch. Intern. Med.,* 153**,** 2093-2101.

Mcewen, B. S. & Seeman, T. (1999). Protective and Damaging Effects of Mediators of Stress: Elaborating and Testing the Concepts of Allostasis and Allostatic Load. *Annals of the New York Academy of Sciences,* 896**,** 30-47.

Mckenzie, S. & Deane, E. M. (2005). Faecal corticosteroid levels as an indicator of well-being in the tammar wallaby, Macropus eugenii. *Comp Biochem Physiol A Mol Integr Physiol,* 140**,** 81-7.

Mendel, C. M. (1989). The free hormone hypothesis: a physiologically based mathematical model. *Endocr Rev,* 10**,** 232-74.

Merl, S., Scherzer, S., Palme, R. & Möstl, E. (2000). Pain causes increased concentrations of glucocorticoid metabolites in horse feces. *Journal of Equine Veterinary Science* 20**,** 586-590.

Metrione, L. C. & Harder, J. D. (2011). Fecal corticosterone concentrations and reproductive success in captive female southern white rhinoceros. *General and Comparative Endocrinology,* 171**,** 283-292.

Metrione, L. C., Penfold, L. M. & Waring, G. H. (2007). Social and spatial relationships in captive southern white rhinoceros (Ceratotherium simum simum). *Zoo Biology,* 26**,** 487-502.

Miller, M., Buss, P., Joubert, J., Mathebula, N., Kruger, M., Martin, L., Hofmeyr, M. & Olea-Popelka, F. (2013). Use of Butorphanol during immobilization of free-ranging White Rhinoceros (*Ceratotherium Simum*). *Journal of Zoo and Wildlife Medicine,* 44**,** 55-61.

Miller, M., Hobbs, N. & Sousa, M. (1991). Detecting stress responses in Rocky Mountain bighorn sheep (Ovis canadensis canadensis) : Reliability of cortisol concentrations in urine and feces. *Can. J. Zool,* 69**,** 15-24.

Miller, M., Kruger, M., Kruger, M., Olea-Popelka, F. & Buss, P. (2016). A scoring system to improve decision making and outcomes in the adaptation of recently captured white Rhinoceroses (Ceratotherium Simum) to captivity. *Journal of Wildlife Diseases***,** S78-S85.

Millspaugh, J., Burke, T., Van Dyk, G., Slotow, R., Washburn, B. & Woods, R. (2007). Stress response of working Africa elephant to transportation and Safari adventures. *Journal of wildlife Management,* 71**,** 1257-1260.

Millspaugh, J. J. & Washburn, B. E. (2004). Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen Comp Endocrinol,* 138**,** 189-99.

Millspaugh, J. J., Washburn, B. E., Milanick, M. A., Beringer, J., Hansen, L. P. & Meyer, T. M. (2002). Non-Invasive Techniques for Stress Assessment in White-Tailed Deer. *Wildlife Society Bulletin,* 30**,** 899-907.

Moberg, G. (2000). Biological response to stress: Implications for animal welfare. *In:* MOBERG, G. & MENCH, J. (eds.) *The biology of animal stress: Basic principles and implications for animal welfare.* Oxon, UK: CABI Publishing.

Monfort, S. L., Mashburn, K. L., Brewer, B. A. & Creel, S. R. (1998). Evaluating adrenal activity in African wild dogs (Lycaon pictus) by fecal corticosteroid analysis. *J Zoo Wildl Med,* 29**,** 129-33.

Morgan, K. N. & Tromborg, C. T. (2007). Sources of stress in captivity. *Applied Animal Behaviour Science,* 102**,** 262-302.

Morley, J., Benton, D. & Solomon, G. (1991). The role of stress and opioids as regulators of the immune response. *In:* MCCUBBIN, J., KAUFMANN, P. & NEMEROFF, C. (eds.) *Stress, Neuropeptides and Systemic Disease,.* San Diego, CA: Academic.

Mostl, E. & Palme, R. (2002). Hormones as indicators of stress. *Domest Anim Endocrinol,* 23**,** 67-74.

Munck, A., Guyre, P. M. & Holbrook, N. J. (1984). Physiological Functions of Glucocorticoids in Stress and Their Relation to Pharmacological Actions. *Endocrine Reviews,* 5**,** 25-44.

Nagata, S., Takeda, F., Kurosawa, M., Mima, K., Hiraga, A., Kai, M. & Taya, K. (1999). Plasma adrenocorticotropin, cortisol and catecholamines response to various exercises. *Equine Veterinary Journal,* 31**,** 570-574.

Nukina, H., Sudo, N., Aiba, Y., Oyama, N., Koga, Y. & Kubo, C. (2001). Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. *Journal of Neuroimmunology,* 115**,** 46-52.

Ott, S., Soler, L., Moons, C. P. H., Kashiha, M. A., Bahr, C., Vandermeulen, J., Janssens, S., Gutiérrez, A. M., Escribano, D., Cerón, J. J., Berckmans, D., Tuyttens, F. a. M. & Niewold, T. A. (2014). Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. *Research in Veterinary Science,* 97**,** 124-128.

Owen-Smith, N. (1971). Territoriality in the white rhinoceros (Ceratotherium simum) Burchell. *Nature,* 231**,** 294-296.

Palme, R. (2005). Measuring Fecal Steroids: Guidelines for Practical Application. *Annals of the New York Academy of Sciences,* 1046**,** 75-80.

Palme, R. & Möstl, E. (1997). Measure of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Int. J. Mamm. Biol,* 62**,** 192-197.

Palme, R., Touma, C., Arias, N., Dominchin, M. & Lepschy, M. (2013). Steroid extraction: get the best out of faecal samples. *Wiener Tierärztliche Monatsschrift– Veterinary Medicine Austria,* 100**,** 238-246.

Peristein, R., Whitnal, M., Abrams, J., Mougey, E. & Reta, R. (1993). Synergistic role of interleukin-6, interleukin-1and tumor necrosis factor in adrenocorticotropin response to bacterial lipopolysaccharide in vitro. *Endocrinology***,** 946-952.

Petersen, H. H., Nielsen, J. P. & Heegaard, P. M. H. (2004). Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary research,* 35**,** 163-187.

Pienaar, D. J. (1994). Habitat preferences of the White Rhino in the Kruger National Park. *Proceedings of a Symposium on "Rhinos as game Ranch Animals", Onderstepoort, 9 & 10 September 1994***,** 59-64.

Pinter-Wollman, N., Isbell, L. A. & Hart, L. A. (2009). Assessing translocation outcome: Comparing behavioral and physiological aspects of translocated and resident African elephants (Loxodonta africana). *Biological Conservation,* 142**,** 1116-1124.

Powell, G. F., Brasel, J. A. & Blizzard, R. M. (1967a). Emotional deprivation and growth retardation simulating idiopathic hypopituitarism. I. Clinical evaluation of the syndrome. *N Engl J Med,* 276**,** 1271-8.

Powell, G. F., Brasel, J. A., Raiti, S. & Blizzard, R. M. (1967b). Emotional deprivation and growth retardation simulating idiopathic hypopituitarism. II. Endocrinologic evaluation of the syndrome. *N Engl J Med,* 276**,** 1279-83.

Pride, E. R. (2005). High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (Lemur catta). *Biology Letters,* 1**,** 60-63.

Pruett, S. B. (2003). Stress and the immune system. *Pathophysiology,* 9**,** 133-153.

Råberg, L., Grahn, M., Hasselquist, D. & Svensson, E. (1998). On the adaptive significance of stress-induced immunosuppression. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 265**,** 1637-1641.

Romano, M. C., Rodas, A. Z., Valdez, R. A., Hernández, S. E., Galindo, F., Canales, D. & Brousset, D. M. (2010). Stress in Wildlife Species: Noninvasive Monitoring of Glucocorticoids. *Neuroimmunomodulation,* 17**,** 209-212.

Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology,* 128**,** 1-24.

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends Ecol Evol,* 19**,** 249-55.

Romero, L. M. (2012). Using the reactive scope model to understand why stress physiology predicts survival during starvation in Galápagos marine iguanas. *General and Comparative Endocrinology,* 176**,** 296-299.

Romero, L. M., Dickens, M. J. & Cyr, N. E. (2009). The reactive scope model — A new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior,* 55**,** 375-389.

Romero, L. M. & Wikelski, M. (2010). Stress physiology as a predictor of survival in Galapagos marine iguanas. *Proceedings of the Royal Society of London B: Biological Sciences,* 277**,** 3157-3162.

Rooney, N. J., Gaines, S. A. & Bradshaw, J. W. S. (2007). Behavioural and glucocorticoid responses of dogs (Canis familiaris) to kennelling: Investigating mitigation of stress by prior habituation. *Physiol Behav,* 92**,** 847-854.

Roth, T. L. & Vance, C. K. (2007). Corticosteroid-induced suppression of in vitro lymphocyte proliferation in four captive rhinoceros species. *J Zoo Wildl Med,* 38**,** 518-25.

Saeb, M., Baghshani, H., Nazifi, S. & Saeb, S. (2010). Physiological response of dromedary camels to road transportation in relation to circulating levels of cortisol, thyroid hormones and some serum biochemical parameters. *Tropical Animal Health and Production,* 42**,** 55-63.

Sahney, S., Benton, M. J. & Ferry, P. A. (2010). *Links between global taxonomic diversity, ecological diversity and the expansion of vertebrates on land*.

Salamano, G., Mellia, E., Candiani, D., Ingravalle, F., Bruno, R., Ru, G. & Doglione, L. (2008). Changes in haptoglobin, C-reactive protein and pig-MAP during a housing period following long distance transport in swine. *The Veterinary Journal,* 177**,** 110-115.

Santymire, R. M., Freeman, E. W., Lonsdorf, E. V., Heintz, M. R. & Armstrong, D. M. (2012). Using ACTH challenges to validate techniques for adrenocortical activity analysis in various African wildlife species. *International Journal of Animal and Veterinary Advances,* 4.

Sapolsky, R. M. (1982). The endocrine stress-response and social status in the wild baboon. *Horm Behav,* 16**,** 279-292.

Sapolsky, R. M. (2010). Physiological and Pathophysiological Implications of Social Stress in Mammals. *Comprehensive Physiology.* John Wiley & Sons, Inc.

Sapolsky, R. M., Romero, L. M. & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev,* 21**,** 55-89.

Sato, M., Ohkawa, R., Yoshimoto, A., Yano, K., Ichimura, N., Nishimori, M., Okubo, S., Yatomi, Y. & Tozuka, M. (2016). Effects of serum amyloid A on the structure and antioxidant ability of high-density lipoprotein. *Bioscience Reports,* 36.

Savetherhino. (2017). *Poaching Statistics* [Online]. London: Save the Rhino International. Available: www.savetherhino.org/rhino_info/poaching_statistics.

Schmidt, A., Hödl, S., Möstl, E., Aurich, J., Müller, J. & Aurich, C. (2010). Cortisol release, heart rate, and heart rate variability in transport-naive horses during repeated road transport. *Domestic Animal Endocrinology,* 39**,** 205-213.

Schook, M. W., Wildt, D. E., Raghanti, M. A., Wolfe, B. A. & Dennis, P. M. (2015). Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceros (Diceros bicornis). *General and Comparative Endocrinology,* 217–218**,** 10-19.

Schwarzenberger, F. (2007). The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *International Zoo Yearbook* 41**,** 52-74.

Soler, L., Gutiérrez, A., Escribano, D., Fuentes, M. & Cerón, J. J. (2013). Response of salivary haptoglobin and serum amyloid A to social isolation and short road transport stress in pigs. *Research in Veterinary Science,* 95**,** 298-302.

Stead, S. K., Meltzer, D. G. & Palme, R. (2000). The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (Loxodonta africana) after ACTH stimulation. *J S Afr Vet Assoc,* 71**,** 192-6.

Sterling, P. (2004). Principles of allostasis: optimal design, predictive regulation, pathophysiology, and rational therapeutics. *Allostasis*.

Steuer, P., Clauss, M., S¸Dekum, K. H., Hatt, J. M., Silinski, S., Klomburg, S., Zimmermann, W., Fickel, J., Streich, W. J. & Hummel, J. (2010). Comparative investigations on digestion in grazing (Ceratotherium simum) and browsing (Diceros bicornis) rhinoceroses. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology,* 156**,** 380-388.

Stratakis, C. A. & Chrousos, G. P. (1995). Neuroendocrinology and pathophysiology of the stress system. *Ann N Y Acad Sci,* 771**,** 1-18.

Terio, K. A., Citino, S. B. & Brown, J. L. (1999). Fecal Cortisol Metabolite Analysis for Noninvasive Monitoring of Adrenocortical Function in the Cheetah (Acinonyx jubatus). *Journal of Zoo and Wildlife Medicine,* 30**,** 484-491.

Termeulen, S. B., Butler, W. R. & Natzke, R. P. (1981). Rapidity of Cortisol Transfer Between Blood and Milk Following Adrenocorticotropin Injection. *Journal of Dairy Science,* 64**,** 2197-2200.

Touma, C. & Palme, R. (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann N Y Acad Sci,* 1046**,** 54-74.

Tridelta. (2017a). *Haptoglobin* [Online]. Ireland: Tridelta Developement. Available: http://www.trideltaltd.com/Haptoglobin-Main-Page.html.

Tridelta. (2017b). *Serum Amyloid A* [Online]. Ireland: Tridelta Developement LTD. Available: http://www.trideltaltd.com/Serum-Amyloid-A-Assay-Kit.html.

Turner, J. W., Jr., Tolson, P. & Hamad, N. (2002). Remote assessment of stress in white rhinoceros (Ceratotherium simum) and black rhinoceros (Diceros bicornis) by measurement of adrenal steroids in feces. *J Zoo Wildl Med,* 33**,** 214-21.

Valle, E., Zanatta, R., Odetti, P., Traverso, N., Furfaro, A., Bergero, D., Badino, P., Girardi, C., Miniscalco, B., Bergagna, S., Tarantola, M., Intorre, L. & Odore, R. (2015). Effects of competition on acute phase proteins and lymphocyte subpopulations – oxidative stress markers in eventing horses. *Journal of Animal Physiology and Animal Nutrition***,** n/a-n/a.

Vance, C. K., Kennedy-Stoskopf, S., Obringer, A. R. & Roth, T. L. (2004). Comparative studies of mitogen- and antigen-induced lymphocyte proliferation in four captive rhinoceros species. *J Zoo Wildl Med,* 35**,** 435-46.

Viljoen, J. J., Ganswindt, A., Palme, R., Reynecke, H. C., Du Toit, J. T. & Langbauer Jr, W. R. (2008). Measurement of concentrations of faecal glucocorticoid metabolites in free-ranging African elephants within the Kruger National ParK. *Koedoe,* 50**,** 18-21.

Von Der Ohe, C. G. & Servheen, C. (2002). Measuring Stress in Mammals Using Fecal Glucocorticoids: Opportunities and Challenges. *Wildlife Society Bulletin,* 30**,** 1215-1225.

Washburn, B. E. & Millspaugh, J. J. (2002). Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *Gen Comp Endocrinol,* 127**,** 217-22.

Wasser, S. K., Bevis, K., King, G. & Hanson, E. (1997). Noninvasive Physiological Measures of Disturbance in the Northern Spotted Owl. *Conservation Biology,* 11**,** 1019-1022.

Wasser, S. K., Hunt, K. E., Brown, J. L., Cooper, K., Crockett, C. M., Bechert, U., Millspaugh, J. J., Larson, S. & Monfort, S. L. (2000). A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *Gen Comp Endocrinol,* 120**,** 260-75.

Wechsler, B. (1995). Coping and coping strategies: a behavioural view. *Applied Animal Behaviour Science,* 43**,** 123-134.

Weingrill, T., Gray, D. A., Barrett, L. & Henzi, S. P. (2004). Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. *Horm Behav,* 45**,** 259-69.

Whitten, P. (1997). Noninvasive methods for the study of stress, reproductive function, and aggression. *Am. J. Phys. Anthropol,* 24**,** 239.

Wikgren, M., Maripuu, M., Karlsson, T., Nordfjäll, K., Bergdahl, J., Hultdin, J., Del-Favero, J., Roos, G., Nilsson, L.-G. & Adolfsson, R. (2012). Short telomeres in depression and the general population are associated with a hypocortisolemic state. *Biological psychiatry,* 71**,** 294-300.

Windle, R. J., Wood, S. A., Shanks, N., Lightman, S. L. & Ingram, C. D. (1998). Ultradian Rhythm of Basal Corticosterone Release in the Female Rat: Dynamic Interaction with the Response to Acute Stress. *Endocrinology,* 139**,** 443-450.

Wingfield, J. C. & Romero, L. M. (2010). Adrenocortical Responses to Stress and Their Modulation in Free-Living Vertebrates. *Comprehensive Physiology.* John Wiley & Sons, Inc.

Wingfield, J. C. & Sapolsky, R. M. (2003). Reproduction and resistance to stress: when and how. *J Neuroendocrinol,* 15**,** 711-24.

Wingfield, J. C., Vleck, C. M. & Moore, M. C. (1992). Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. *Journal of Experimental Zoology,* 264**,** 419-428.

Wong, A. W., Bonde, R. K., Siegal-Willott, J., Stamper, M. A., Colee, J., Powell, J. A., Reid, J. P., Deutsch, C. J. & Harr, K. E. (2012). Monitoring oral temperature, heart rate, and respiration rate of West Indian manatees (Trichechus manatus) during capture and handling in the field. *Aquatic Mammals,* 38**,** 1.

Ye, R. D. & Sun, L. (2015). Emerging functions of serum amyloid A in inflammation. *Journal of Leukocyte Biology,* 98**,** 923-929.

Young, K. M., Walker, S. L., Lanthier, C., Waddell, W. T., Monfort, S. L. & Brown, J. L. (2004). Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *Gen Comp Endocrinol,* 137**,** 148-65.